Kindlin-1 Protein-Protein Interactions and Functional Relevance to Kindler Syndrome

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Abstract

Kindler syndrome (KS) is an autosomal recessive genodermatosis resulting from pathogenic mutations in the *FERMT1* (*KIND*) gene. This gene encodes kindlin-1 (also known as fermitin family homologue 1), a focal adhesion protein involved in activation of the integrin family of extracellular matrix receptors. Most cases of KS show a marked reduction or complete absence of the kindlin-1 protein in keratinocytes, resulting in defective integrin activation and abnormal cell adhesion and migration. However, currently very little is known about the way in which different *FERMT1* mutations found in patients impact upon keratinocyte behaviour. The aim of this thesis is to screen for novel binding partners of kindlin-1 to elucidate possible new functions for this protein in epidermal cells. The main approaches taken were to assess differentially expressed proteins in normal vs. KS patient keratinocytes and also to use recombinant wild type and mutant kindlin-1 for pulldown assays to identify novel binding partners. Mass spectrometry analysis revealed a number of downregulated proteins in KS keratinocytes including epidermal growth factor receptor (EGFR) and thrombospondin-1 (TSP-1). Immunoblotting confirmed a significant reduction in both of these proteins. Further investigation showed changes in localisation of both TSP-1 and EGFR, and also defective signalling and response following EGF stimulation in KS keratinocytes. Moreover, EGFR levels in KS keratinocytes were partially restored following treatment with lysosomal inhibitors, suggesting that kindlin-1 can regulate the recycling of this receptor. Mass spectrometry analysis also identified the potential interaction partners of kindlin-1 to be talin and integrin linked kinase (ILK). Biochemical analysis revealed that kindlin-1 interacts specifically with the talin head domain. This interaction was significantly reduced with a mutant form of kindlin-1

identified in a KS patient that lacks part of the F3 subdomain. Taken together, these studies have identified novel proteins that are regulated by kindlin-1 and may be important regulators of keratinocyte adhesion and migration.

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Abbreviations

ADAM – A disintegrin and metalloproteinase

ADP – adenosine diphosphate

APS – ammonium persulphate

ATP – adenosine tri-phosphate

BM – basement membrane

BSA – bovine serum albumin

CCD – charged couple device

cDNA – complementary deoxyribonucleic acid

CHO – Chinese hamster ovary cells

DEJ – dermal-epidermal junction

DMEM – Dulbecco's modified Eagle's medium

DMSO – dimethyl sulphoxide

DNA – deoxyribonucleic acid

dNTP – deoxyribonucleotide triphosphate

EB – epidermolysis bullosa

E-cadherin – epithelial cadherin

EDTA - ethylenediaminetetra-acetic acid

ECM – extracellular matrix

EGF – epidermal growth factor

EGFR – epidermal growth factor receptor

EMT – epithelial-mesenchymal transition

F-actin – filamentous actin

FACS – fluorescence-activated cell sorting

FAK – focal adhesion kinase

FBS – fetal bovine serum

FC – flow cytometry

FERM – four point one protein, ezrin, radixin and moesin

FLIM – fluorescence-lifetime imaging microscopy

FRET – fluorescence resonance energy transfer

GAG – glycosaminoglycans

GAPDH – glyceraldehyde 3-phosphate dehydrogenase

GFP – green fluorescent protein

GFRs – growth factor receptors

GST – glutathione S-transferase

GTPases – guanosine triphosphate-binding proteins

HEK293T – human embryonic kidney 293 T-antigen

HMEC – human mammary epithelial cells

HPV – human papilloma virus

HSC70 – heat shock cognate 71 kDa protein

ICAM – intercellular adhesion molecule

IF – immunofluorescence

ILK – integrin linked kinase

IP – immunoprecipitation

IPTG – isopropyl β-D-1-thiogalactopyranoside

kDa – kilodaltons

KS – Kindler syndrome

LAD-III – leukocyte adhesion deficiency-III

LB – Luria broth

LDL – low-density lipoprotein

LDV – leucine-aspartic acid-valine

MAPK – mitogen-activated protein kinase

MD – membrane distal

mins – minutes

MMP – matrix metalloproteinase

Mn – manganese

MP – membrane proximal

mRNA – messenger ribonucleic acid

NHK – normal human keratinocyte

NHS – normal human skin

NLS – nuclear localisation signal

PBS – phosphate buffered saline

PFA – paraformaldehyde

PI – protease inhibitor

PIP2 - Phosphatidylinositol 4,5-bisphosphate

PIP3 - Phosphatidylinositol 3,4,5-trisphosphate

PH – pleckstrin homology

PTB – phophotyrosine-binding

PX – phox domain

Rap1 – ras-related protein 1

RFP – red fluorescent protein

RGD – arginine-glycine-aspartic acid

RIAM – Rap1-GTP-interacting adaptor molecule

ROCK1 – Rho-associated coiled-coil containing protein kinase 1

RTK – receptor tyrosine kinase

SDS – sodium dodecyl sulphate

SDS-PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis

S.E.M – standard error of the mean

SFM – serum free media

SH2 – src homology 2 domain

SILAC – stable isotope labelling by amino acids in cell culture

siRNA – small interfering ribonucleic acid

SNX – sorting nexin

S.O.C. – super optimal broth with catabolite repression

TBS – tris buffered saline

TFR – transferrin receptor

TSP – thrombospondin

UNC-112 – uncoordinated protein 112

VCAM – vascular cell adhesion molecule

WB – western blotting

WT – wild type

ZO – zona occludens

Chapter 1: Introduction

1.1 Structure of the skin

1.1.1 Epidermis and epidermal keratinocytes

The skin is the largest organ of the human body. It consists of a multi-layered stratified epidermis, a dermis containing collagen and elastic fibres, and underlying subcutaneous fat. The epidermis is a stratified squamous epithelium. It forms a protective barrier against the environment and has several important functions including; prevention of water loss, excluding toxins, resisting mechanical stress and involvement in immune responses. The epidermis is divided into 4 main layers which are the basal layer (stratum basale), spinous layer (stratum spinosum), granular layer (stratum granulosum) and cornified layer (stratum corneum) (Simpson *et al.*, 2011). The basal layer consists of a single layer of specialised skin epidermal cells known as keratinocytes, which are attached to an underlying matrix. The cells proliferate and some of their daughter keratinocytes enter the spinous layer, where they exit the cell cycle, grow larger and establish intercellular connections. Cells in the granular layer flatten and assemble a water-impermeable cornified envelope underlying the plasma membrane. And lastly the corneal layer keratinocytes release lysosomal enzymes to degrade major organelles; the cells become completely squamous and are tightly crosslinked together to form the cutaneous barrier (Simpson *et al.*, 2011). The structure and composition of the stratum corneum and upper epidermis form a physical barrier against the external environment and contribute to an anti-microbial innate immune system (Gallo and Nizet, 2008).

1.1.2 The dermis

The dermis is the layer that lies between the epidermis and subcutaneous tissues; it consists of connective tissue and buffers the body from stress and strain. It is subdivided into two layers, the papillary region and a deep thicker area known as the reticular dermis. The dermis is tightly connected to the epidermis through a basement membrane and contains structural components including collagen, elastic fibres, and ground substance (extrafibrillar matrix). Of the twenty seven types of collagen found in vertebrates, eight of these are expressed in the skin. Collagen not only maintains structure but also has important functions such as cell adhesion, chemotaxis and migration (Myllyharju and Kivirikko, 2004). Elastic fibres are composed of elastin and fibrillin proteins, and are less tough than collagen but provide extensible properties to the skin. This allows the skin to stretch and recoil without damage (Midwood and Schwarzbauer, 2002). The ground substance is the intercellular material in which the cells and fibres are embedded and is mostly composed of proteoglycans and glycoproteins. The proteoglycans are made up of multiple core proteins which have large molecules, glycoaminoglycans, such as hyaluronic acid. These molecules play an essential role in water retention (Heinegård, 2009). The fibroblast is the predominant cell type in the dermis and secretes collagens, elastic fibres and ground substance. Other cells in the dermis include mast cells, plasma cells, lymphocytes, dermal dendritic cells and histiocytes. The dermis also contains mechanoreceptors that provide the sense of touch and heat, hair follicles, sweat glands, sebaceous glands, apocrine glands, lymphatic vessels and blood vessels. These blood vessels provide nourishment and waste removal for both dermal and epidermal cells.

1.1.3 The subcutis

The subcutis is the innermost layer of the skin, and is composed of adipose tissue. The adipose tissue is compartmentalised by fibrous septae. Bundles of fibres from the dermis extend into the subcutis to strengthen the connection between these two compartments. This layer functions as an insulator, conserving the body's heat, and as a shock-absorber, protecting the inner organs. It also stores fat as an energy reserve for the body. The blood vessels, nerves, lymph vessels, and hair follicles also cross through this layer. It also has an endocrine function, communicating with the hypothalamus via secreted molecules such as leptin to alter energy expenditure and appetite.

1.1.4 Structure of the dermal-epidermal junction

The junction between the dermis and the epidermis is composed of a specialised layer of extracellular matrix (ECM) called the basement membrane, and is referred to as the dermal-epidermal junction (DEJ) in the skin. The DEJ restricts molecules based on their size and charge and also mediates cells to migrate under normal or pathological conditions (Burgeson and Christiano, 1997). The DEJ has two main classes of extracellular macromolecules including polysaccharide chains of the class called glycosaminoglycans (GAGs), which are usually found covalently linked to protein in the form of proteoglycans, and fibrous proteins, including collagen, elastin, fibronectin, and laminin, which have both structural and adhesive functions. These proteins can bind to each other to make a highly crosslinked extracellular matrix (LeBleu *et al.*, 2007). Ultrastructurally, the DEJ can be divided into four zones and this includes the cell membranes of the basal keratinocytes, which

contains the hemidesmosomes and integrin based adhesions (Burgeson and Christiano, 1997). The second zone is the lamina lucida, through which the anchoring filaments traverse, is rich in laminin. The lamina densa is the third zone and is mainly composed of collagen IV but also consists of laminins and proteoglycans. The final zone is the sub-basal lamina, which contains anchoring fibrils, mostly collagen VII (Burgeson and Christiano, 1997).

The basal keratinocytes of the epidermis assemble hemidesmosomes and integrin based adhesions at the basal surface to maintain cell attachment to the underlying basement membrane (BM) (McMillan *et al.*, 2003). Hemidesmosomes link the keratin intermediate filaments of basal keratinocytes with the ECM while integrins provide transmembrane connections from the ECM to the intracellular actin network. Hemidesmosomal structures are composed of α6β4, plectin, bullous pemphigoid antigen 1e (BP230) and BP180, which mediate linkage between keratin filaments such as keratin 5 and 14 to collagen fibres and laminin in the BM (McMillan *et al.*, 2003; Fassihi *et al.*, 2006). The adhesion sites are complex molecular structures that connect ECM proteins such as fibronectin, collagen and laminin to the actin cytoskeleton via integrin receptors. Integrins do not bind to actin directly but are connected through various actin-binding proteins such as talin, paxillin, vinculin and actinin. The only focal adhesion protein that is associated with a disease is kindlin-1, whose pathogenesis results in Kindler syndrome (Siegel *et al.*, 2003; Tsuruta *et al.*, 2011). Loss of function mutations in any of the components of the hemidesmosome including the keratin filaments and ECM proteins result in dysadhesion of the epidermis and development of blisters between the keratinocyte layers of the skin and dermis (McMillan *et al.*, 2003; Fassihi *et al.*, 2006). These phenotypes have been identified as pathogenic in a family of skin fragility and blistering disorders

know as epidermolysis bullosa (EB). There are several subtypes of this disorder, which includes EB simplex, junctional EB and dystrophic EB, and are defined by the gene that is mutated and the severity of the condition (Fassihi *et al.*, 2006; Tsuruta *et al.*, 2011). Molecules at the BM are also involved in cell signalling between the dermis and the epidermis. In addition to its role in maintaining dermal-epidermal adhesion, the BM prevents the epidermal keratinocytes from invading the dermis (Amano, 2009). It also determines polarity for the epidermis so that once the BM is assembled, the basal keratinocytes recognise the surface adjacent to the BM as its basal surface (Amano, 2009).

1.1.4.1 Components of the ECM

Among the structural components of the ECM, many transiently secreted proteins also exist including members of the matricellular family. Thrombospondin-1 (TSP-1) is a member of the matricellular family and was originally discovered as a major component in platelet α-granules. It is secreted into the matrix, binds to matrix components and to cell surface receptors, but does not play a structural role. TSP-1 is a protein with many functions, which it achieves through its multiple domain structure (Adams and Lawler, 2004). TSP1 binds to extracellular matrix ligands including fibrinogen, fibronectin, some collagens, latent and active transforming growth factor-beta-1, MMPs, and heparan sulphate proteoglycans. TSP1 also binds to cell surface receptors including CD36, CD47, and the integrins alpha-V/beta-3, alpha-3/beta-1, alpha-4/beta-1, and alpha-6/beta-1 (Chen *et al.*, 2000). TSP1 in a context-dependent and cell-specific manner stimulates or inhibits cell adhesion, proliferation, motility, and survival (Chen *et al.*, 2000). TSP-1 has been shown to be

involved in thrombus formation (Murphy-Ullrich and Mosher, 1985) and plays a significant role in wound healing with increased expression seen at the wound edge (Reed *et al.*, 1993). It is also a potent inhibitor of angiogenesis, demonstrated by its ability to inhibit endothelial cell migration (Short *et al.*, 2005) and in addition has been shown to modulate tumour biology through cell adhesion, migration and invasion. However, it appears to have roles in both tumour growth and inhibition (Esemuede *et al.*, 2004). The ability of TSP-1 to bind different matrix proteins and cell surface receptors may account for its multi-functional and sometimes opposing nature.

1.1.5 Epidermal stem cells

There are three differentiating epithelial compartments in the skin, which includes the epidermis, hair follicles and associated glands such as the sebaceous gland. The growth of both the epidermis and sebaceous gland is continuous. In the epidermis, proliferation occurs in the basal layer and the terminally differentiated progeny move through the suprabasal layer to the cell surface (Blanpain and Fuchs, 2009). Cutaneous stem cells are slow-cycling cells and are found in the bulge of the hair follicles and also in the sebaceous glands. Stem cells niches contribute to regeneration of the skin and lineage-specific differentiation. (Ghazizadeh and Taichman, 2001). The stem cells are involved in the regeneration of the hair follicles, sebaceous glands and also the interfollicular epidermis during wound healing processes. The stem cells within the basal layer of keratinocytes are responsible for the production of the majority of the interfollicular epidermis. The stem cells in the bulge of the hair follicles are characterised by high levels of β integrin, keratin 15

and keratin 19 expression (Jones and Simons, 2008). Stem cells divide asymmetrically to give rise to one daughter cell committed to terminal differentiation and another daughter stem cell (Lechler and Fuchs, 2005). There are, however, several modes of asymmetrical division, for example one that positions the mitotic spindle parallel to the BM and another that places the division perpendicular to the BM. β1 integrin mediated adhesions are known to regulate differentiation of epidermal keratinocytes, while Notch signalling has been implicated as a key factor that regulates the basal to spinous switch (Blanpain and Fuchs, 2009). A highly proliferative population of cells can be generated by some stem cells and are known as transient amplifying cells. These cells are found within clusters of stem cells and are characterised by positive expression of β1 integrin, Notch ligand delta, cell surface proteoglycan MSPG, desmosomal protein desmoglein 3 (DSG3) and transcription factor LRIG1 (Jones and Simons, 2008).

1.2 Kindler syndrome

1.2.1 History of Kindler syndrome

Kindler syndrome (KS; OMIM 173650) is caused by an abnormality in the actin cytoskeleton and its association with the ECM due to a deficiency or defect in the focal adhesion protein, kindlin-1 (also known as fermitin family homologue 1 and previously kindlerin) (Jobard *et al.*, 2003; Siegel *et al.*, 2003). KS was first described by Theresa Kindler in 1954. The patient, a 14 year old girl, presented with congenital blistering on the arms, legs and feet and also developed pigmentary changes after suffering from photosensitivity (Kindler, 1954). The clinical features of KS overlapped with those seen in other diseases such as the inherited skin blistering disease EB. Similarities to hereditary acrokeratotic poikilodema, an autosomal

dominant disorder described by Weary et al. resulted in the term Weary-Kindler syndrome being introduced (Weary *et al.*, 1971). This led to confusion in providing a clear diagnosis but subsequent reports tended to separate autosomal recessive KS from autosomal dominant Weary syndrome. Further molecular characterisation of the overlapping clinical disorders allowed KS to be classified as a distinct and separate genodermatosis.

1.2.2 Clinical features of Kindler syndrome

The initial feature of KS is trauma-induced blistering which reduces or ultimately stops in later childhood. Patients also develop progressive poikiloderma and skin atrophy which is more acute on the dorsal aspects of the hands and feet. In addition, KS patients may show marked photosensitivity (Ashton, 2004). Other features include early onset destructive periodontal disease and severe desquamative gingivitis causing bleeding gums and loss of teeth. Mucosal involvement including anal, vaginal, urethral and oesophageal stenoses is frequently seen in individuals with KS. Patients can also develop gastrointestinal symptoms such as severe colitis. Finger webbing, squamous cell carcinoma and nail dystrophy are other reported features (Ashton, 2004; Lai-Cheong *et al.*, 2007; Lai-Cheong *et al.*, 2010). Some of the clinical features of KS are illustrated in Figure 1.1.

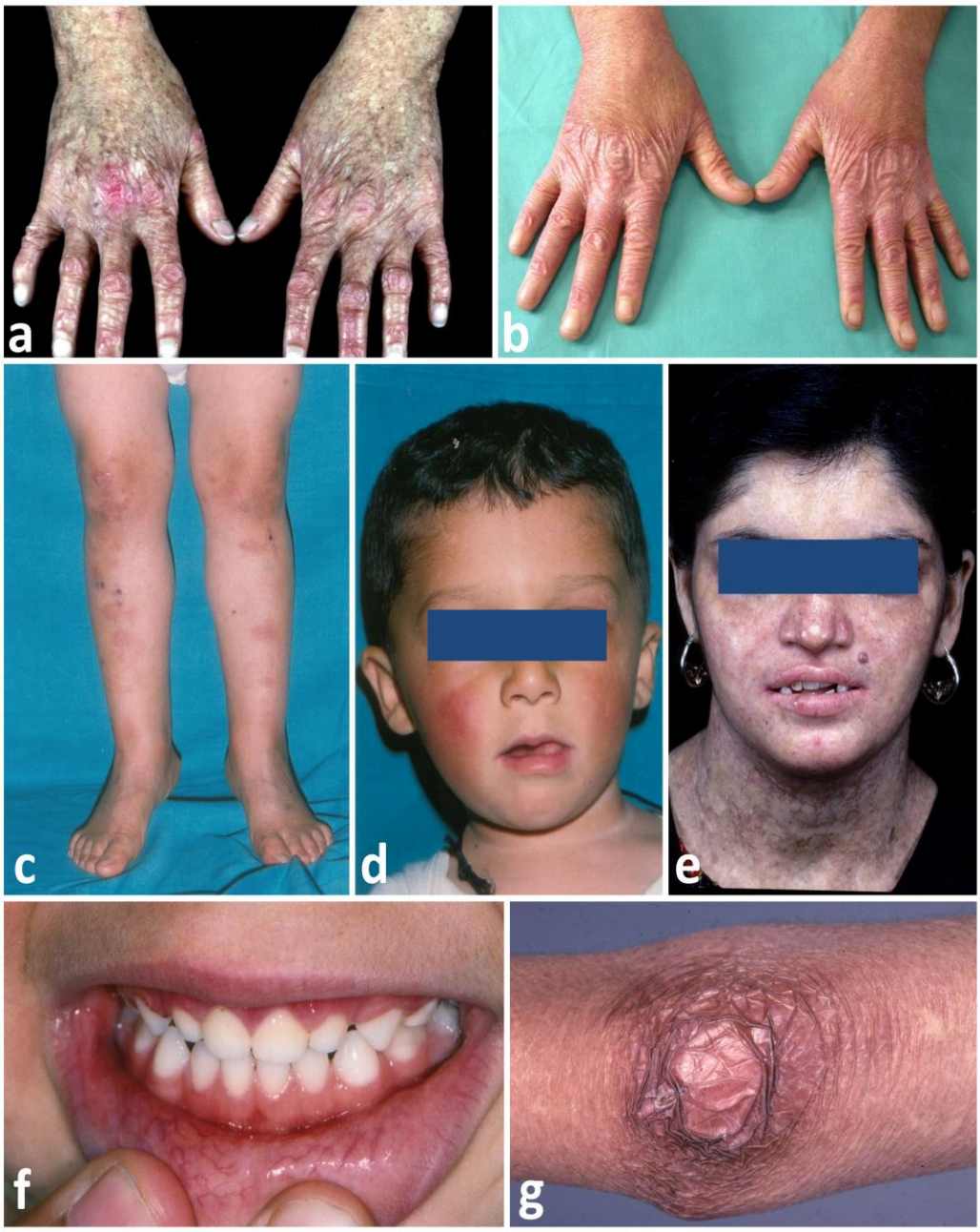


Figure 1.1: Clinical features of Kindler syndrome.

a) Significant poikiloderma on dorsa of the hands. b) Atrophy and erythema on the dorsae of the hands. c) Trauma-induced scars and post-inflammatory hyperpigmentation on the lower limbs. d) Facial erythema. e) Marked poikiloderma on the face and neck. f) Telangiectasiae on lower lip mucosa and mild oral inflammation. g) Skin wrinkling and atrophy on the elbow.

1.2.3 Histological abnormalities in Kindler syndrome skin

Histologically, characterisation of KS skin shows that the epidermis is flattened and atrophic with hyperkeratosis as well as a loss of rete ridges. There is vacuolisation of the basal layer of the epidermis, telangiectatic vessels as well as pigmentary incontinence in the superficial dermis (Hovnanian *et al.*, 1989; Shimizu *et al.*, 1997). Electron microscopy reveals multiple planes of cleavage, marked reduplication of the lamina densa and cleft formation in the lamina lucida. Abnormal cleavage may be within or just above the basal layer of epidermis, within the lamina lucida or below the lamina densa. Hemidesmosomes and anchoring fibrils, however, appear grossly normal (Shimizu *et al.*, 1997). Figure 1.2 highlights some of the structural aspects seen in KS skin such as poikiloderma and reduplication of the basement membrane.

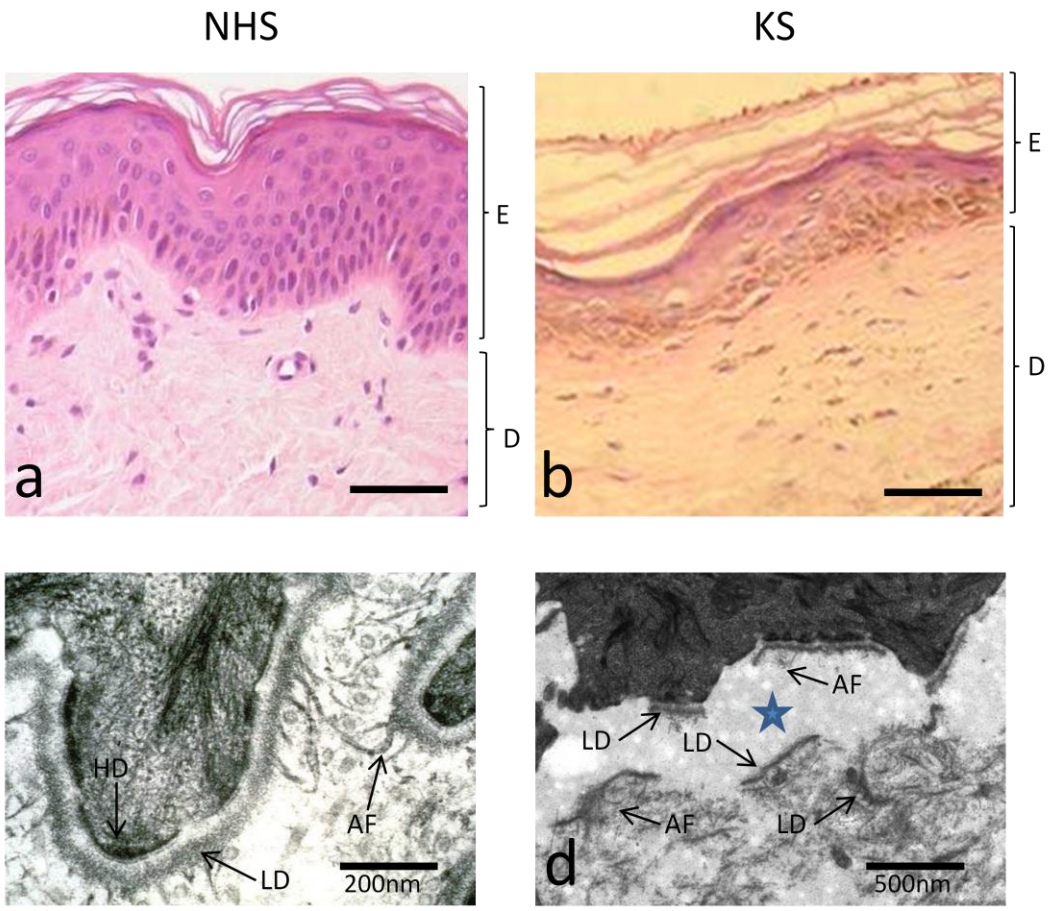


Figure 1.2: Structure of Kindler syndrome skin

Light microscopy (LM) of a) normal skin and b) KS skin. E = epidermis; D = dermis Scale bar = 50 μm. c) Ultrastructural appearances of the DEJ in NHS: HD = hemidesmosome; LD = lamina densa; AF = anchoring fibrils. d) in KS, there is a blister (asterisk). In addition, the LD is fragmented with some present in the roof and base. In some parts of the section AF are seen below the LD in the blister roof, while in others parts of the section the AF are below the LD in the floor of the blister. Fragmentation of the LD and duplication of the LD (shown within the dermis) are characteristic ultrastructural hallmarks of KS. The plane of blistering is variable in KS: here the cleavage is both sub-LD and also within the lamina lucida, while other cases may reveal some blistering within basal keratinocytes (not shown here).

1.2.4 Identification of the Kindler syndrome gene

The gene implicated in KS was identified in 2003 by two independent groups (Jobard *et al.*, 2003; Siegel *et al.*, 2003). Genome-wide linkage analysis was performed and showed the gene responsible for KS to be on chromosome 20p12.3. Six predicted genes were identified within this region, but only one contained a mutation; *FLJ20116*, which is also known as *c20orf42* and subsequently renamed *KIND1* (Siegel *et al.*, 2003). Other nomenclatures associated with this gene include unc-related protein 1 (*URP1*), *UNC112A* and fermitin family homologue 1 (*FERMT1*), however, for the purposes of the present work it will be referred to as *KIND1*.

The *KIND1* gene spans 48.5 kb of genomic DNA and contains 14 coding (exons 2 to 15) and one non-coding exon (exon 1). The *KIND1* gene is the human homologue of the Caenorhabditis elegans gene, *unc-112*, which encodes a protein that mediates actin cytoskeleton anchorage with the ECM (Rogalski *et al.*, 2000). The human *KIND1* mRNA transcript is ~4.9 kb and gene expression was shown in cultured keratinocytes, colon, kidney and placenta and at lower expression levels in heart, skeletal muscle, liver, and small intestine (Siegel *et al.*, 2003). The colon contains an additional transcript that is ~5.8 kb. Since identifying the *KIND1* gene, 150 patients and 46 mutations have been reported. These mutations include nonsense, frameshift, splice site and internal deletion changes (Has *et al.*, 2011b). The *KIND1* gene encodes the protein kindlin-1 which consists of 677 amino acid residues and has a molecular weight of 77 kDa (Siegel *et al.*, 2003). The kindlin-1 protein possesses a bipartite FERM (4.1 protein, ezrin, radixin, moesin) domain at the C-terminal region. The FERM domain consists of three subdomains, F1, F2 and F3 as shown in Figure 1.3. The F2 subdomain is disrupted by a pleckstrin homology (PH) domain. Both the

FERM and PH domain are present in many proteins that are involved in anchorage of the cytoskeleton to the ECM and the PH domain is found in several cell signalling molecules that associate with cell membranes by binding to specific lipid molecules (Meves *et al.*, 2009).

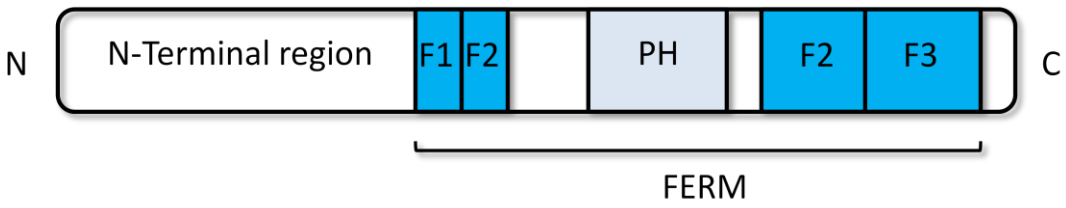


Figure 1.3: Domain organisation of kindlin-1 protein

The kindlin-1 protein consists of a bipartite FERM (Four point one protein, Ezrin, Radixin and Moesin) domain, which contains three subdomains, denoted F1, F2 and F3. There is a PH (Pleckstrrin homology) sandwiched within the F2 subdomain.

1.3 Kindlin-1

1.3.1 Kindlin-1 homologues

The kindlin-1 protein has closely related homologues in fish (*Onchrhynchus*), fruit flies (*Drosophila melanogaster*) and worms (*Caenorhabditis elegans*) (Rogalski *et al.*, 2000; Schaller, 2000; Siegel *et al.*, 2003). In *C.elegans* the homologue of kindlin-1 is the unc-112 protein and loss of this protein results in a paralysed lethal phenotype (pat – paralysed arrested elongation at twofold). This is characterised by a loss of adhesion between muscle and the ECM during embryo unfolding (Rogalski *et al.*, 2000). Embyros with a loss of perlecan (unc-52, α integrin (pat-2) or β integrin

(pat-3) subunits also exhibit a similar PAT phenotype, indicating that UNC-112 may be involved in cell-ECM interactions at sites of integrin adhesions (Rogalski *et al.*, 2000).

1.3.2 Kindlin-1 localisation in skin and keratinocytes

An anti-kindlin-1 antibody has been used to determine the localisation of kindlin-1 in normal human and KS patient skin as well as in keratinocytes. In normal skin, kindlin-1 localises mostly in the epidermis, more specifically in keratinocytes and also along the DEJ. However in KS skin, the signal was markedly reduced and almost completely absent from the basal keratinocytes (Siegel *et al.*, 2003). In keratinocytes, kindlin-1 colocalised with filamentous actin as well as with vinculin, a marker for focal adhesions (Siegel *et al.*, 2003). The localisation of kindlin-1 in these cells is supportive of a role in anchorage of the actin cytoskeleton to the ECM.

1.3.3 Kindlin-1 and keratinocyte behaviour

In vitro studies have demonstrated that kindlin-1-deficient keratinocytes display a number of abnormal phenotypes. Initial studies using siRNA to deplete kindlin-1 expression in HaCaT cells showed a notable decrease in cell spreading (Kloeker *et al.*, 2004). In addition to this, Herz et al. reported that keratinocytes derived from KS patients had multiple narrow leading edges with multipolar shapes (Herz *et al.*, 2006). This was in contrast to the polygonal shapes and polarised lamelipodia of NHKs. Furthermore, KS keratinocytes had smaller cell areas, showed reduced proliferation, and demonstrated reduced adhesion on fibronectin and laminin-332.

Wound closure assays also showed that these keratinocytes had reduced migration with reduced persistence. However, KS keratinocytes did not show any defects in apoptosis (Herz *et al.*, 2006). These results were substantiated by a later report showing similar findings (Has *et al.*, 2008). Taken together, these findings provide some insight into the atrophic and blistering skin phenotype seen in KS but the physiological functions of kindlin-1 and the molecular disease mechanisms in KS still remain unclear.

1.3.4 Kindlin-1 and intestinal disease

Patients with KS also present with gastrointestinal problems such as bowel inflammation and diarrhoea. Kindlin-1 localises to the plasma membrane of epithelial cells in the colon and rectum (Kern *et al.*, 2007). Loss of kindlin-1 leads to multiple focal detachments of the epithelium from the underlying tissue in intestinal mucosa, which results in breach of the epithelial barrier and to an inflammatory intestinal phenotype (Kern *et al.*, 2007). Studies using kindlin-1 knockout mice showed severe epithelial atrophy, as seen in KS patients, however the mice died shortly after birth. This was a result of detachment of intestinal epithelial cells induced by shear force, significant inflammation as well as organ destruction (Ussar *et al.*, 2008). Furthermore, *in vitro* studies have shown a considerable reduction in integrin activation which caused a defect in adhesion of cells to the ECM resulting in detachment (Ussar *et al.*, 2008). Collectively, these findings suggest that the intestinal phenotypes seen in KS patients are not a secondary effect, but rather occur as a direct result of kindlin-1 deficiency.

1.3.5 Kindlin-1 and cancer

Kindlin-1 may potentially play a role in cancer development as reports have discovered a significant increase in kindlin-1 expression levels in particular tumours. Kindlin-1 was shown to be overexpressed in 60% of lung cancers and 70% of colon cancers, indicating a close association (Weinstein *et al.*, 2003). Subsequent microarray studies performed on human mammary epithelial cells (HMEC) treated with TGF-β show an increase in kindlin-1 expression level. TGF-β is found to be overexpressed in many colon cancers and the increase in kindlin-1 levels may play a role in epithelial to mesencymal transition (EMT) a crucial aspect of cancer formation as a result of its effect on cell migration and spreading (Kloeker *et al.*, 2004). Kindlin-1 may also be involved in breast cancer progression as it has been found to be differentially expressed in breast tumours and it is significantly increased in tumours metastasising to the lung (Sin *et al.*, 2011). By contrast, a recent report suggests that kindlin-1 has an opposing role by inhibiting tumour growth and invasion in lung cancer (Zhan *et al.*, 2012). In addition, several clinical case reports have shown an increased incidence of squamous cell carcinoma in KS patients, however the mechanism underlying this has yet to be elucidated (Lai-Cheong *et al.*, 2009b).

1.4 Kindlin-1 and integrins

1.4.1 Regulation of integrin activation

Integrins are a major class of cell adhesion receptor family. They are hereterodimeric transmembrane proteins consisting of α and β subunits, which consist of a large extracellular domain, a transmembrane domain and a short cytoplamic domain

(Hynes, 2002; Humphries *et al.*, 2006). Integrins mediate the attachment of cells to the ECM, providing a link between the ECM ligands and the cytoskeleton. Integrins also transduce a variety of signalling pathways that regulate various cellular processes, including proliferation, survival, shape, polarity, motility, gene expression and differentiation. These processes are important in development, haemostasis, immune responses and also cancer formation (Hynes, 2002). As integrins have no inherent kinase activity and cannot associate with the cytoskeleton directly, they require adaptor proteins to enable the receptor to connect to F-actin and mediate transduction of vital signals into the cell (Harburger and Calderwood, 2009). Integrins convey important information regarding the local environment, location, adhesive state and surrounding matrix, in this way the cell can respond accordingly with responses such as migration, survival and differentiation. Binding of integrins to ECM ligands can trigger their clustering and formation of multi-protein complexes, which involve an array of different scaffold and signalling proteins (Harburger and Calderwood, 2009).

Both talin and kindlins have been shown to bind directly to the cytosplasmic domain of β integrin subunits and regulate integrin activation, as shown in Figure 1.4. Kindlin-1 was implicated in integrin regulation as a result of studies performed on KS keratinocytes which showed defects in migration, spreading and adhesion (Kloeker *et al.*, 2004; Herz *et al.*, 2006; Has *et al.*, 2008). These factors were indicative of a potential disruption to integrin signalling. Moreover, there is a significant reduction in integrin activation although total expression levels are normal in KS keratinocytes (Lai-Cheong *et al.*, 2009a). Kloeker et al. then showed evidence that kindlin-1 in fact is a constituent of integrin focal adhesions and is able to form complexes with both β1 and β3 integrin cytoplasmic tails (Kloeker *et al.*,

2004). Kindlin-1 FERM (4.1 protein, ezrin, radixin, moesin) domain has been shown to bind directly to β1 and β3 subunits. Kindlin-1 has been shown to bind to the β integrin cytoplasmic tail (Harburger *et al.*, 2009). More specifically the F3 subdomain of kindlin-1 is required for this interaction as mutants generated in this region including the tryptophan to alanine (Trp612Ala) disrupted direct interaction with integrins (Harburger *et al.*, 2009).

Overexpression of a GFP-tagged kindlin-1 in KS keratinocytes was shown to restore integrin activation and rescue the KS cellular phenotype (Lai-Cheong *et al.*, 2009a). Using GST pull down assays, kindlin-1 has been shown to bind directly to the cytoplasmic tails of β1 and β3 integrins (Ussar *et al.*, 2006; Harburger *et al.*, 2009). This interaction occurs between the second conserved NxxY motif (795 Tyr), a preceding threonine-containing region (788 Thr and 789 Thr) of the integrin β1A tail, and a conserved tryptophan in the F3 subdomain of the kindlin-1 FERM domain (612 Trp) (Harburger *et al.*, 2009). Furthermore, kindlin-1 can also interact with migfilin and kindlin-2 (Lai-Cheong *et al.*, 2008) as well as with α-actinin and focal adhesion kinase (Has *et al.*, 2009). It has recently been shown, using fluorescence-activated cell sorting, that transient expression of kindlin-1 (and also of kindlin-2) in Chinese hamster ovary (CHO) cells is able to inhibit the activation of endogenous α5β1 or stably expressed αIIbβ3 integrins in these cells (Harburger *et al.*, 2009). This occurs independently of direct kindlin–integrin interaction since mutant kindlin with impaired integrin binding ability also inhibits integrin activation (Harburger *et al.*, 2009). However, co-expression of kindlin-1 (or kindlin-2) with the talin head domain results in activation of αIIbβ3 but not β1 integrin, suggesting that kindlin-1 and -2 may exert integrin-specific effects (Harburger *et al.*, 2009). The mechanisms by

which kindlin-1 may control integrin activation other than by direct association remain unknown.

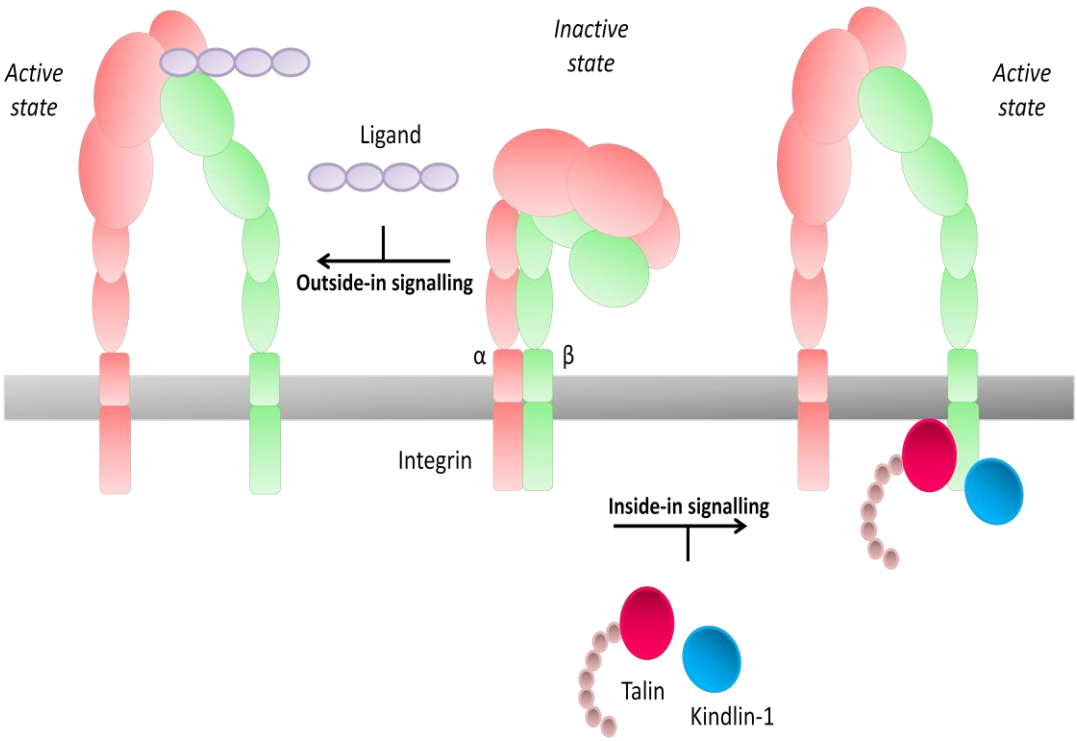


Figure 1.4: Integrin activation

Model showing bi-directional signalling of integrin. Outside-in signalling occurs as a result of ligand binding to the extracellular domain of integrin leading to conformational change and activation. While inside-out signalling occurs when kindlin and talin bind to the β integrin subunit resulting in integrin activation.

1.4.1.1 Talin

Talin is a major structural protein and was the first protein to be found to activate integrins. There are two talins in humans, talin 1 and talin 2. Talin 1 is ubiquitously expressed while talin-2 is more restricted in its expression, and is found primarily in striated muscle and in the brain (Senetar *et al.*, 2007). Similarly to kindlins, talin belongs to the FERM domain family of proteins, a domain that is conserved in many cytoskeletal-associated proteins involved in localising proteins to the plasma membrane. The FERM domain is further divided into subdomains of F1, F2 and F3. The talin protein consists of a N-terminus head domain of ~50 kDa which contains the FERM arranged in a linear form, in contrast to other FERM proteins (Calderwood, 2004; Elliott *et al.*, 2010); it also has a rod domain of ~ 220 kDa at the C-terminus. The talin head has been shown to directly interact with the β integrin subunit cytoplasmic tail (Calderwood *et al.*, 1999). The F3 subdomain has the highest affinity for the β tail and resembles a phosphotyrosine binding (PTB) domain which binds to the membrane proximal NPxY motif (Calderwood *et al.*, 1999; Calderwood *et al.*, 2002) and a membrane proximal helix of the β integrin subunit tail (Wegener *et al.*, 2007). The expression of the head domain alone results in activation of the integrin β3 six times higher than that of full length talin due to masking of the binding site (Yan *et al.*, 2001). The rod domain of talin consists of an actin binding site which allows it to link integrins to the actin cytoskeleton. Talin also has a number of vinculin binding sites in the rod domain which is important for recruiting vinculin to adhesion sites (Critchley, 2009). Talin is autoinhibited by binding of the α-helical component of the rod domain to the F3 subdomain. This is released by calpain cleavage which increases binding of the talin head domain to the β3 integrin subunit (Yan *et al.*, 2001). Martel et al also reported that interaction with

phosphatidylinositol 4, 5-bisphosphate (PIP2) can significantly increase binding of talin to β1 integrins (Martel *et al.*, 2001). Additionally, talin activation of integrin is enhanced is through the Ras-related protein 1 (Rap1)-Rap1–GTP-interacting adapter molecule (RIAM) pathway (Anthis and Campbell, 2011). The proposed model of activation of integrins by talin is dependent on the interaction between the talin F3 subdomain and the membrane proximal β tail which results in the disruption of the α and β tails association (Calderwood, 2004). Talin forms a salt bridge with the β tail and for full activation induces the reorientation of the β subunits transmembrane domain (Anthis *et al.*, 2009). The contribution of kindlins to integrin activation, however, is still unclear. Although considerable evidence suggests that kindlins enhance integrin activity, there is conflicting data which shows that it can also inhibit activation (Harburger *et al.*, 2009); thus further investigation is needed to resolve these issues.

1.4.2 Integrin ligands and function

The human genome contains 8 β and 18 α integrin subunits, which combine to give 24 different combination of integrin receptors, that provide specificity to ligands and have nonredundant roles (Hynes, 2002) . Integrins are expressed abundantly in all nucleated cells and they are also expressed simultaneously in the cells. Knockout of integrins in mice show a wide range of phenotypes including a block in perimplantation, embryonic or perinatal lethality and major developmental defects (Hynes, 2002; Gahmberg *et al.*, 2009). Leukocyte adhesion deficiency syndrome (LAD-1) is a good example of mutations in integrins effects on humans. In this particular case the disease occurs due to mutation in the β2 integrin gene leading to impaired functions of leukocytes resulting in microbial infections, impaired wound

healing, defects in phagocytosis and chemotaxis (Gahmberg *et al.*, 2009). There are two main types of integrins expressed in the basal epidermis in skin and these are α6β4 and α3β1. The integrin α6β4 is critical for the assembly of hemidesmosomes and mutations in either subunit results in junctional EB with skin blistering (Hashmi and Marinkovich, 2011). Furthermore, the integrin α3β1 is important for cell-ECM adhesion as well as cell-cell adhesion, and mutations in the α3 subunit has been shown to also cause EB, albeit a new form of EB that has not previously been characterised (Has *et al.*, 2012). The development of these skin fragility disorders highlight the importance of integrins in skin homeostasis.

Integrins bind to a number of ligands and can be divided into four main classes dependent on the integrin-ligand combination which considers the structural basis of the molecular interaction. The four classes include the arginine-glycine-aspartic acid (RGD)-binding integrins, the leucine-aspartic acid-valine (LDV)-binding integrins, the A-domain β1 integrins and the non-αA domain-containing laminin-binding proteins (Humphries *et al.*, 2006). The RGD motif is a tripeptide sequence that binds the interface between the α and β subunit. The integrins that bind this motif are especially promiscuous, in particular β3 integrin which binds to a large number of ECM and soluble vascular ligands (Humphries *et al.*, 2006). The LDV-binding integrins bind to ligands containing the acidic motif LDV including fibronectin. The A-domain β1 integrins form a distinct collagen/laminin binding subfamily. The α subunits of this family contain the A (Willebrand factor or inserted (I)) domain, which is the principal domain required for ligand binding in the class (Humphries *et al.*, 2006; Gahmberg *et al.*, 2009). Finally, the non-αA domain-containing laminin-binding proteins include α3β1, α6β1, α7β1 as well as α6β4, which are all highly specific laminin receptors. Other integrin ligands identified are the ADAM family of

metalloproteases, E-cadherin, fibrillin, vitronectin, ICAM, MMPs, TSP and VEGF (Humphries *et al.*, 2006). The connection between integrins and ligands in the underlying matrix helps the cells to endure pulling forces, thus providing a mechanical role in anchorage. It also provides structural stability to the epithelium, and connects the ECM to the cell cytoskeleton, which allows force-transmission.

1.5 Kindlin-2 and -3

1.5.1 Kindlin-2

The kindlin protein family consists of three members; kindlin-1, kindlin-2 and kindlin-3 (Siegel *et al.*, 2003). All of the kindlin proteins have similar protein architecture comprising a FERM domain at the C-terminus with a PH domain inserted in the F2 subdomain, as shown in figure 1.3. Kindlin-2 was the first of the family members to be discovered when Wick et al. in 1994 performed a differential cDNA library screen to try and identify proteins involved in G0/1 to S phase progression (Wick *et al.*, 1994). This approach revealed a number of proteins, one of which they termed as mitogen inducible gene-2 (Mig-2) (Wick *et al.*, 1994). Siegel et al. subsequently identified Mig-2 as having close homology to kindlin-1 (~62%) and renamed the protein kindlin-2 (also known as fermitin family homologue 2) (Siegel *et al.*, 2003). Kindlin-2 was found to be ubiquitously expressed and is the only member of its family found to be expressed in embryonic stem cells. Similar to kindlin-1, kindlin-2 localises to focal adhesions and is additionally also located at cell-cell junctions (Ussar *et al.*, 2006). It localises in the nuclei of certain cell types, including smooth muscle cells (Kato *et al.*, 2004), and is the only family member to possess a nuclear localisation signal (NLS) (Ussar *et al.*, 2006). Kindlin-2 has many similar features to kindlin-1 including its ability to bind the membrane distal motif

integrins β1 and β3 cytoplasmic tails and cause activation via its F3 subdomain (Montanez *et al.*, 2008; Harburger *et al.*, 2009). Furthermore, it interacts with migfilin, which mediates its localisation to focal adhesion sites. This also serves as a link to the actin cytoskeleton as migfilin interacts with filamin, an actin binding protein (Tu *et al.*, 2003). Kindlin-2 also binds to ILK which was initially demonstrated with unc-112 in *C.elegans* and later shown for the human homologue kindlin-2 (Mackinnon *et al.*, 2002; Montanez *et al.*, 2008). This demonstrates that kindlin-2 is a bi-directional signalling molecule as it contributes to both outside-in signalling and inside-out signalling of integrins.

1.5.1.1 Kindlin-2 and embryonic lethality

To analyse the functional relevance of kindlin-2, the impact of kindlin-2 deficiency was reported in mice by two independent groups. They both showed that kindlin-2 knock-out mice died during embryogenesis before or at embryonic age 7.5 as a result of severe detachment of the epiblast and endoderm causing peri-implantation lethality (Dowling *et al.*, 2008a; Montanez *et al.*, 2008). The age of lethality was similar to that reported for β1 integrin and ILK knockout mice (Dowling *et al.*, 2008a; Montanez *et al.*, 2008). Furthermore, embryonic stem cells with kindlin-2 deficiency displayed reduced compactness of stem cell colonies as well as reduced adhesion to ECM substrates including laminin-111, laminin-332 and fibronectin. The cells also had a marked absence of active β1 integrin, measured by loss of 9EG7 staining, an antibody that recognises the active state of this protein (Montanez *et al.*, 2008). Downregulation of kindlin-2 in zebrafish also caused premature lethality, further highlighting the functional importance of the protein (Dowling *et al.*, 2008a).

The lack of a disease model of kindlin-2 in humans, however, may be explained by the fact that it is vitally important during early development.

1.5.1.2 Kindlin-2 and cancer

Kindlin-2 has been implicated in carcinogenesis, showing increased expression levels in the aggressive breast cancer cell line TMX2-28. Silencing of kindlin-2 in this cell line led to a significant reduction in cell invasion (Gozgit *et al.*, 2006). A recent study also showed overexpression of kindlin-2 in a number of breast cancer cell lines, including Hs578T and MDA-MB-231. In these cell lines, kindlin-2 was shown to regulate breast cancer proliferation, apoptosis and survival to induce tumour formation (Zhao *et al.*, 2013). Kindlin-2 is suggested to cause genome instability as there was an increase in mitotic abnormalities including an increased number of chromosomal break fragments, chromatid breaks and gaps (Zhao *et al.*, 2013). Furthermore, kindlin-2 overexpression has been associated with a number of other highly invasive cancers, including malignant mesothelioma (MM) tumour (An *et al.*, 2010), a small subset of high grade invasive bladder cancer (Talaat *et al.*, 2011) and in gastric cancer tissues (Shen *et al.*, 2012). In contrast to these findings, kindlin-2 has also shown to play an important role in the suppression of mesenchymal cancer cell invasion (Shi and Wu, 2008). Moreover, kindlin-2 levels were found to be lower in the invasive uterine leiomyosarcoma cells compared to the higher expression in leiomyoma cells which are less invasive (Kato *et al.*, 2004). Taken together, kindlin-2 has shown variable levels of expression in human cancers and the expression level of kindlin-2 may serve as a prognostic marker for tumour progression in certain cancers.

1.5.1.3 Kindlin-2 and muscle development

The loss of kindlin-2 in *C.elegans* resulted in severe muscle detachment which highlighted its important role in integrin mediated events. Furthermore, integrins as well as ILK play a critical role in cardiac muscle (Hannigan *et al.*, 2007). These key observations led to further investigations into the role of UNC-112 vertebrate homologue in cardiac development. Kindlin-2 was chosen as the target as it is the only member of the kindlin family to be expressed in the cardiac and skeletal muscle (Dowling *et al.*, 2008a). Kindlin-2 knockdown has been achieved in zebrafish using anti-sense morpholino, which resulted in severe disruption in cardiac structure and function (Dowling *et al.*, 2008a). The embryos developed pericardial oedema and had significantly slower mean heart rates in comparison to wild type embryos. There was disruption to the intercalated discs and the ventricular size, shape and contractility was affected (Dowling *et al.*, 2008a). Finally, knockdown of kindlin-2 caused skeletal myopathy demonstrating an important role for kindlin-2 in skeletal myogenesis (Dowling *et al.*, 2008b).

1.5.2 Kindlin-3

The final member of the kindlin family is kindlin-3, also known as unc-related protein 2 or fermitin family homologue 3. Kindlin-3 expression is more restricted compared to the other kindlin proteins; it is mostly expressed in haematopoietic cells, including leukocytes and erythrocytes, with particularly high levels in platelets and megakaryocytes (Siegel *et al.*, 2003; Pasini *et al.*, 2006; Ussar *et al.*, 2006). Additionally, kindlin-3 has also recently been found to be expressed in endothelial cells (Bialkowska *et al.*, 2010). The importance of kindlin-3 was highlighted in a study which produced knockout of kindlin-3 in mice. Mice homozygous for kindlin-

3 knockout died within a week of birth, and presented with osteopetrosis, severe bleeding in gastrointestinal tract, skin, brain and bladder which was determined to be due to severe platelet dysfunction (Moser *et al.*, 2008). Loss of kindlin-3 in platelets showed a lack of β1 and β3 integrin activation, as well as a failure to form lamellipodia and spread (Moser *et al.*, 2008). Overexpression of kindlin-3 in a macrophage cell line confirmed the ability of kindlin-3 to cause integrin activation. The F-3 PTB domain of kindlin-3 activates integrins by binding to the membrane distal NxxY motif of both β1 and β3 integrin cytoplasmic tails (Moser *et al.*, 2008; Yates *et al.*, 2012a). In addition to its role in β1 and β3 integrin activation in platelets, kindlin-3 was also shown to be involved in β2 activation in leukocytes. In leukocytes, activation of β2 integrins facilitates cell adhesion to endothelial cells, allowing transmigration through the blood vessel wall. As with the other integrin subunits, kindlin-3 mediates β2 activation through interaction with the distal NxxY domain of the cytoplasmic tail (Moser *et al.*, 2009a).

1.5.2.1 Kindlin-3 and LAD-III syndrome

Leukocyte adhesion deficiency type III (LAD-III) syndrome (OMIM 612840) is a rare hereditary human disease (also known as LAD-I variant). It is characterised by a failure to activate β1, β2 and β3 integrins in haematopoietic cells resulting in severe bleeding due to impaired platelet activation and frequent bacterial and fungal infections due to a defect in leukocyte adhesion to endothelial cells (Moser *et al.*, 2009a). The genetic cause of this disease was discovered to be due to mutations in the *KIND3* gene (Mory *et al.*, 2008; Kuijpers *et al.*, 2009; Malinin *et al.*, 2009; Svensson *et al.*, 2009). Patients with LAD-III syndrome can also exhibit osteopetrosis, an increased density and hardening of the bones, which is also seen in

kindlin-3 deficient mice (Schmidt *et al.*, 2011). The osteoclasts in these mutant mice have defective integrin activation, and they have an inability to spread and organise the actin cytoskeleton. In addition, the cells cannot form podosomes and sealing zones and are unable to degrade bone matrix. The cells exhibited a loss of integrin activation due to kindlin-3 deficiency, highlighting the importance of this process in osteoclast mediated bone resorption (Schmidt *et al.*, 2011).

1.5.2.2 Kindlin-3 and erythrocyte function

Kindlin-3 is also expressed in erythrocytes (Pasini *et al.*, 2006), with a potential role in maintaining the membrane skeleton (Kruger *et al.*, 2008). Scanning electron microscopy showed that kindlin-3-deficient erythrocytes had highly irregular shapes and sizes with membrane invaginations and protuberances (Kruger *et al.*, 2008). The use of a quantitative proteomics approach known as SILAC (stable isotope labelling by amino acids in cell culture) followed by mass spectrometry analysis revealed differences in expression of a number of membrane skeleton proteins. Results showed a complete absence of ankyrin-1, band 4.1, adducin-2 and dematin (Kruger *et al.*, 2008). These findings showed that kindlin-3 plays a significant role in the assembly of membrane skeletal proteins that maintain the structural integrity of erythrocytes.

1.6 Cell adhesion and migration

1.6.1 Integrin-based adhesions

Integrin-mediated adhesion and migration are essential for a number of important biological processes such as immune responses, tissue regeneration and repair,

embryonic development, as well as pathological events in cancer and inherited immunodeficiency disorders. Integrin-mediated adhesions continually form and turn-over to function as signalling centres that coordinate a network of signalling pathways and provides linkage between the ECM and the actin cytoskeleton (Huttenlocher and Horwitz, 2011). There are four subclasses of adhesions that have been identified; nascent adhesions, focal complexes, focal adhesions and fibrillar adhesions (Geiger and Yamada, 2011; Huttenlocher and Horwitz, 2011; Scales and Parsons, 2011). Nascent adhesions and focal complexes are small dot-like structures that form at the protrusions of lamellipodia. The nascent adhesions can either disassemble or mature into focal complexes which have a longer life and are induced by activated Rac (Huttenlocher and Horwitz, 2011; Scales and Parsons, 2011). Focal adhesions are much larger in size and also have a slower turnover, with an elongated morphology. They are the best characterised and consist of integrin receptors and a multiprotein complex of structural and signalling proteins which are linked to the actin cytoskeleton (Huttenlocher and Horwitz, 2011). Fibrillar adhesions are stable contacts which are also elongated in shape; they are found prominently under the central areas of cells and form parallel to bundles of fibronectin (Geiger and Yamada, 2011; Scales and Parsons, 2011).

1.6.2 Focal adhesion proteins

Integrin-based adhesions link the ECM to the cell actin cytoskeleton generating force and allowing the cell to respond to its environment. Integrins lack catalytic activity and actin binding properties, and hence there is a need for adaptor proteins which mediate integrin activation and act as a base for assembly of larger signalling and structural scaffolds (Legate and Fassler, 2009). There are 3 main categories of

integrin adaptors; first are the adaptor proteins with structural functions, including talin and filamin. These proteins connect integrins to the cytoskeleton as they are able to bind directly to F-actin. Secondly, there are adaptor proteins with scaffolding function that provide binding sites for multiple focal adhesion proteins. Thirdly, there are adaptor proteins with enzymatic activity which propagate signal transduction pathways and include focal adhesion kinase (FAK), Src and integrin linked kinase (ILK) (Legate and Fassler, 2009).

Talin is one example of a structural protein that is recruited early to adhesion sites, and the phosphorylation of tyrosine residues on the NPxY motif of β integrin tail regulates its binding which facilitates integrin activation. The FERM domain not only binds β integrin tails but also F-actin, PIP2 and FAK. There are additional binding sites for β integrin tail and F-actin, and three vinculin binding sites on the rod domain of talin. It acts as structural protein, either directly or indirectly, via vinculin, connecting integrins to actin (Legate and Fassler, 2009).

Vinculin and paxillin are examples of scaffolding adaptor proteins. Vinculin is activated after binding with talin and helps to increase integrin clustering and focal adhesion growth (Humphries *et al.*, 2007). Paxillin also binds to numerous proteins, thus providing the ability to control important events such as adhesion turnover and cell migration. (Harburger and Calderwood, 2009). Another example of a structural adaptor protein is filamin which interacts with β integrin tail and also has an N-terminal actin binding domain which is involved in actin organisation, thus coupling integrins to the actin cytoskeleton directly (Das *et al.*, 2011). Filamin binding to β integrin tail was shown to inhibit cell migration and membrane protrusions and is now widely accepted as a negative regulator of integrin activation (Calderwood *et al.*, 2001; Das *et al.*, 2011; Kim *et al.*, 2011).

FAK is an example of an adaptor protein with catalytic function. This protein tyrosine kinase is important for adhesion turnover, Rho-family GTPase activation, cell migration and cross talk between growth factor signalling and integrins. Integrin activation and clustering leads to activation of FAK which can also be enhanced by growth factor receptor co-stimulation. When FAK is activated, it autophosphorylates and binds Src kinase which in turn phosphorylates other sites on FAK and also FAK-binding proteins. FAK binds to downstream signalling proteins such as paxillin, Grb2 and PI3K. Thus, FAK can mediate multiple signalling events that regulate growth, survival and morphogenesis (Schlaepfer *et al.*, 1999; Harburger and Calderwood, 2009). In addition to binding to FAK, Src kinase can directly interact with the tail of β integrin subunits to propagate kinase activity and is involved in the control of cell spreading (Schlaepfer *et al.*, 1999). Another important binding partner of β integrin subunits and downstream signalling molecule is ILK. ILK is recruited to integrin sites by binding to kindlin-2, as the loss of kindllin-2 results in loss of localisation of ILK form focal adhesions (Mackinnon *et al.*, 2002; Montanez *et al.*, 2008). Furthermore, ILK also forms a complex with PINCH and the actin- and paxillin-binding protein PARVIN. In this way, ILK has a key role in integrin signalling and actin cytoskeleton reorganisation (Wickstrom *et al.*, 2010). ILK also plays a role in crosstalk between integrins and receptor tyrosine kinases; for example the association of ILK with Nck-1 protein through PINCH (Hehlgans *et al.*, 2007). Nck-2 is involved in binding and recruiting proteins that regulate receptor tyrosine kinases, thus allowing crosstalk between integrins and EGFR or PDGFR in signalling events implicated in cell survival signalling processes (Ho and Dagnino, 2012). Furthermore, ILK has also recently been shown to participate in keratinocyte

front-rear polarity and migration in response to EGF stimulation and integrin activation (Ho and Dagnino, 2012).

1.6.3 Regulation of F-actin in migration

Actin is the vital component that links protrusion at the leading edge to adhesion assembly, maturation and turnover. During migration, actin drives protrusions by forming different organisations in lamellipodia and filopodia; the two main types of protrusive structures (Stricker *et al.*, 2010). Assembly of functional actin networks requires the coordination of different regulators such as filament crosslinkers, nucleation and elongation factors, and motor proteins. Actin filaments are polarised due to structural polarity with a fast-growing barbed (or plus) ends and slow-growing pointed (or minus) ends (Le Clainche and Carlier, 2008). Monomeric or globular actin (G-actin) with bound ATP polymerises to form F-actin, which may hydrolyse its bound ATP to ADP resulting in depolymerisation (Le Clainche and Carlier, 2008). Actin filaments are organised into bundles or networks by crosslinking proteins resulting in structures like lamellipodia and filopodia. Actin filaments can undergo 'treadmilling' a process whereby the length remains constant as G-actin associates at the plus end and dissociates at the minus end (Le Clainche and Carlier, 2008). In lamellipodia, the small GTPases Cdc42 and Rac activate the nucleation promoting factors Wiskott–Aldrich syndrome protein (WASP) and WASP-family verprolin-homologous protein (WAVE) which in turn activate the Arp2/3 complex. The Arp2/3 complex is a nucleation factor that promotes the generation of actin filament branches, and results in a broad dendritic-like actin network (Pollard and Borisy, 2003; Jaffe and Hall, 2005). In filopodia, Cdc42 propagates linear actin polymerisation through the diaphanous-related formins and

vasodilator-stimulated phosphoprotein (VASP). Organisation of polymerised actin into elongated filaments is conducted by fascin (Mattila and Lappalainen, 2008).

1.6.4 Growth factors in adhesion and migration

Growth factors are proteins that bind to specific growth factor receptors (GFRs) to stimulate cellular growth, proliferation and differentiation (Streuli and Akhtar, 2009). Growth factors are produced by many different cell types. The membrane receptors for growth factors are expressed on all cells and most cells have receptors for more than one growth factor, thus allowing more synergistic signalling by multiple growth factors. In adherent cells, the action of both cell adhesion and GFRs are needed for optimal output of synergistic signalling (Streuli and Akhtar, 2009; Ivaska and Heino, 2011). Integrins are able to regulate growth factor and GFRs, and vice versa, since growth factors can influence the function of integrins by modulating expression. For example fibroblast growth factor-2 (FGF-2) can upregulate α5β1 expression in endothelial cells, while TGF-β1 is able to increase β1 and β5 levels and promote the synthesis of αvβ6 in keratinocytes (Streuli and Akhtar, 2009). Integrins are also able to bind to growth factors directly and cause subsequent activation of GFR downstream signalling pathways. For example, αvβ6 can bind to the RGD motif on latent transforming growth factor-β (TGF-β) leading to activation of the receptor (Munger and Sheppard, 2011). Furthermore, α3β1 and αvβ3 can recognise vascular endothelial growth factor-A (VEGF-A) which regulates endothelial cell migration, an important process in angiogenesis (Hutchings *et al.*, 2003).

The expression, localisation or post-translational modifications of GFRs and levels of growth factors can all be regulated by integrins and the receptors also share many

downstream signalling molecules. There are several mechanisms by which integrins and GFRs cross-talk and are able to coordinate signals (Ivaska and Heino, 2011). One mechanism is collaborative activation, which can enable growth factor-dependent GFR signalling (Ivaska and Heino, 2011). For example, clustering of integrins can provide the right environment for GFRs to bind downstream signalling proteins after activation through ligand binding (Giancotti and Tarone, 2003). Direct activation of GFRs in the absence of growth factors is another mechanism by which cross-talk can manifest (Ivaska and Heino, 2011). When cells undego integrin-dependent adhesion, it can increase the responsiveness of the cell to growth factors and loss of adhesions can result in growth arrest and anoikis (Frisch and Francis, 1994; Danen and Yamada, 2001; Schwartz and Assoian, 2001). An example of integrin-mediated activation of GFRs is the ability of integrins to induce EGFR phosphorylation in the absence of EGF (Moro et al. 1998). Mechanistically, β1 integrin has been shown to co-immunoprecipitate with EGFR and the clustering of the two receptor types triggers transactivation (Moro *et al.*, 2002; Alam *et al.*, 2007). FAK is phosphorylated after integrin ligand binding and then combines with other signalling proteins such as Src, p130Cas, Shc and Grb2 which transmit integrin-triggered signals to the extracellular signal-regulated kinases (ERK)-mitogen-activated protein (MAP) kinase pathway (Moro *et al.*, 2002). FAK associates with activated GFRs through its N-terminal domain and has important functions in promoting EGF and platelet-derived growth factor (PDGF)-stimulated cell migration (Sieg *et al.*, 2000). Figure 1.5 highlights one of the cross-talk points between integrins and GFRs.

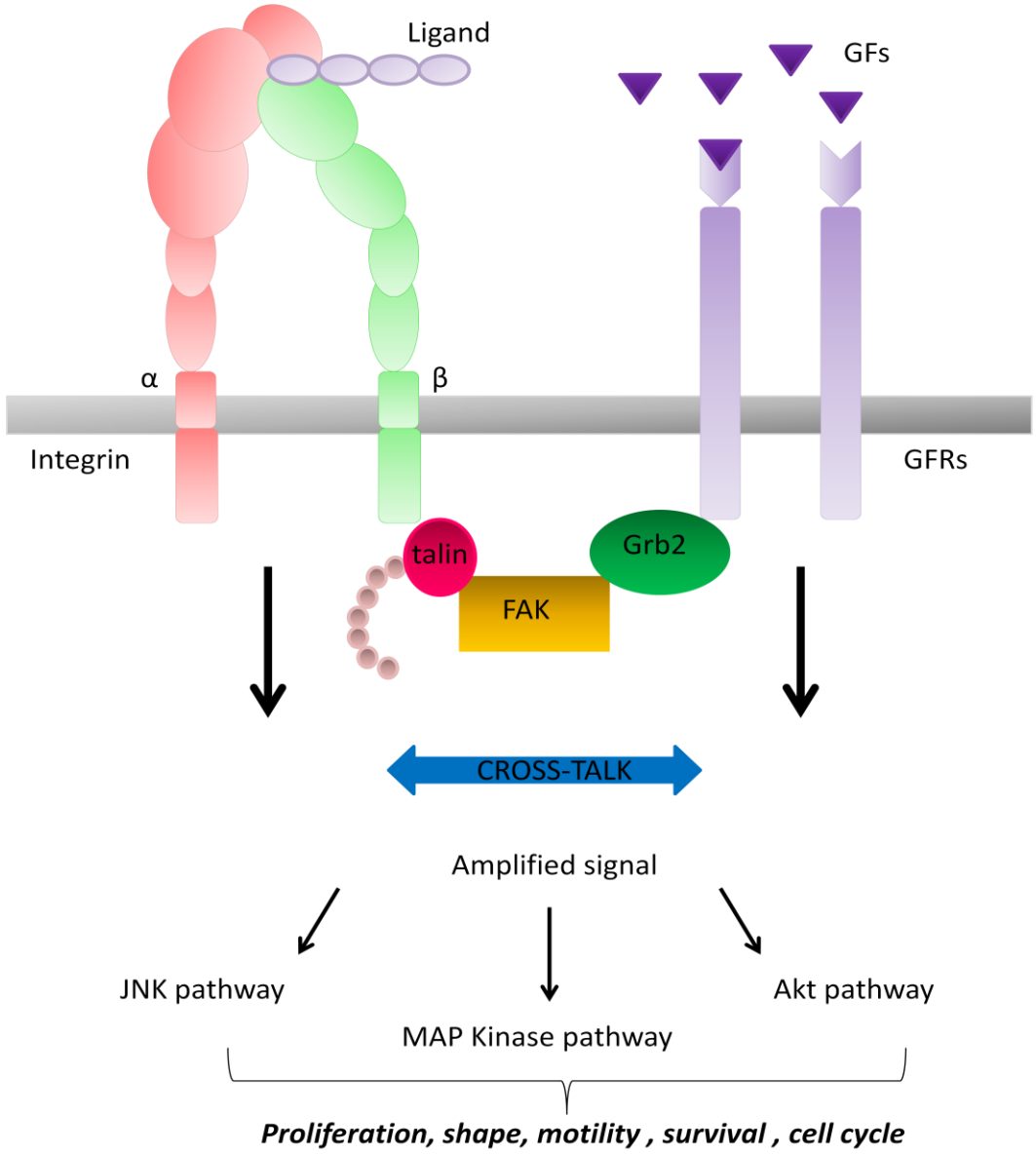


Figure 1.5: Cross-talk between integrins and growth factor receptors

Integrins and growth factor receptors (GFRs) are able to cross-talk potentiating downstream signalling pathways. One convergence point for the two receptors is through FAK, which binds and transmits integrin signals through interactions with talin. FAK can also interact with Grb2 which allows it to transmit GFR signals downstream (Moro *et al.*, 2002). Growth factor and integrin cross-talk leads to amplification of signals leading to changes in proliferation, shape, motility, survival and cell cycle.

1.7 Hypothesis and aims

Kindlin-1 plays an important role in integrin activation but its function in the skin is still not fully understood. It is unclear how loss of kindlin-1 results in the array of phenotypes observed in KS skin and keratinocytes. Therefore, investigations into the molecular and cellular pathology of KS keratinocytes will provide insight into the function of kindlin-1 in both health and disease and help to elucidate its role in integrin-dependent and independent functions.

1.7.1 Hypothesis

Kindlin-1 associates with specific binding partners, both integrin-dependent and independent, resulting in control of keratinocyte cell adhesion and motility.

1.7.2 Aims

- To characterise kindlin-1 regulated proteins in KS keratinocytes.
    - Identify differentially-expressed proteins in normal human keratinocytes (NHK) and Kindler syndrome (KS) cell lysates using mass spectrometry.

- Identify novel binding partners of kindlin-1.
    - Use kindlin-1 GST pulldown analysis and mass spectrometry to identify new potential binding partners for kindlin-1. Investigate these in the context of keratinocyte cell behaviour.

- Characterise a novel KS patient mutation of kindlin-1.
    - Analyse a novel small intragenic deletion mutation in kindlin-1 in a KS patient that may provide new insight into kindlin-1 function or binding partners.

Chapter 2:

Materials and Methods

2.1 Reagents

Table 2.1 Cell culture Reagents

Reagent	***Source***
Bovine Collagen, Type I	BD Biosciences
Calcium Nitrate	Sigma Aldrich
Calyculin A (10 µM final)	Calbiochem
Dymethyl sulphoxide (DMSO)	Sigma
Epidermal Growth Factor (EGF)	Sigma Aldrich
Fetal Bovine Serum	Sera Laboratories International Ltd.
Fugene HD	Promega
High Glucose Dulbecco's modified Eagle's media (DMEM)	Sigma Aldrich
Leupeptin (25 µM final)	Calbiochem
L-Glutamine	PAA
Lipofectamine	Invirogen
MG132 (20 µM final)	Calbiochem
OptiMEM	Gibco
Penicillin/ Streptomycin	Gibco
Phosphate Buffered Saline (PBS)	Lonza
Polybrene (hexadimethrine bromide)	Sigma Aldrich
Protease inhibitor (PI) cocktail set 1 (stock 100X, dilute to 1X) Containing: AEBSF, Hydrochloride - 500 µM Aprotinin, Bovine lung, crystalline – 150 nM E-64 Protease Inhibitor - 1 µM EDTA Disodium – 0.5 mM Leupeptin, Hemisulphate – 1 µM	Calbiochem
Serum-Free Keratinocyte Medium (Keratinocyte-SFM)	Sigma
Trypsin/EDTA	PAA

Table 2.2: Molecular biology Reagents

Reagent	***Source***
Agarose	Sigma Aldrich
Ampicillin	Sigma Aldrich
BL21 (DE3) competent cells	Agilent Technologies
Bovine serum albumin (BSA)	New England Biolabs
Deoxynucleosides (DNTPs)	Bioline
Hyperladder I	Biolabs
Hypperladder V	Biolabs
Kanamycin	Sigma Aldrich
Luria Bertani (LB) agar and broth	Sigma Aldrich
Midiprep kit	Qiagen
Miniprep kit	Qiagen
NEbuffers	New England Biolabs
One Shot TOP10 chemically competent E.Coli	Invitrogen
Phusion HF Buffer (5X)	Finnzymes
Phusion Hot Start DNA polymerase	Finnzymes
Phusion buffer (10X)	New England Biolabs
Phusion polymerase	New England Biolabs
QIAquick gel extraction kit	Qiagen
Quickchange Site-directed mutagenesis kit	Stratagene
Restriction enzymes	New England Biolabs
Safeview	NBS Biologicals
T4 DNA Ligase and Buffer	New England Biolabs
Zero Blunt PCR Cloning Kit	Invitrogen

Table 2.3: Biochemical assay reagents

Reagent	***Source***
1.5 mm Cassettes	Invitrogen
2-mercaptoethanol	Sigma Aldrich
30% Acrylamide/Bis solution	Biorad
A/G agarose affinity matrix	Alpha Diagnostic International
Ammonium persulphate (APS)	Sigma Aldrich
Bovine Serum Albumin	PAA the cell culture company
Bromophenol blue	Sigma Aldrich
Calyculin A	Sigma Aldrich
Coomasie blue	Fluka Chemika
ECL Plus western blotting detection system	GE Healthcare
Fluorsave Mounting media	Calbiochem
Glutathione Sepharose 4 Fast Flow beads	Amersham
Glycine	Sigma Aldrich
Hybond ECL Nitrocellulose membrane	Amersham Biosciences
Immersol 510 Immersion oil	Zeiss
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Sigma Aldrich
Medical X-Ray film	Fuji
Milk Powder	MERCK
Paraformaldehyde (PFA)	Sigma Aldrich
PeqGOLD protein marker V	PeqLab
Pierce BCA protein assay kit	Thermo Scientific
PBS tablets	Oxoid
Pierce ECL western blotting substrate	Thermo Scientific
Ponceau S solution	Sigma Aldrich
Sodium Chloride	Sigma Aldrich
Tetramethylethylenediamine (TEMED)	Sigma Aldrich
Tissue-Tek OCT compound	VWR international
Tris-Base	Sigma Aldrich
Tris-HCL	Sigma Aldrich
Triton X-100	Sigma Aldrich
Tween-20	Sigma Aldrich

Table 2.4: Materials and solutions for biochemical assays

Buffers/Solutions	***Composition***
5% stacking acrylamide gel	5% 30%-acrylamide mix, 125mM Tris-HCl (pH6.8), 0.1% Sodium dodecyl sulphate (SDS), 0.1% ammonium persulphate (APS), 1% TEMED
8% resolving acrylamide gel	8% 30%-acrylamide mix, 400 mM Tris (pH 8.8), 0.1% SDS, 0.1% APS, 0.05% TEMED
12% resolving acrylamide gel	12% 30%-acrylamide mix, 400 mM Tris (pH 8.8), 0.1% SDS, 0.1% APS, 0.05% TEMED
Coomassie staining solution	0.025% coomassie brilliant blue R250, 40%methanol, 7% acetic acid
Destaining solution	50% methanol, 10% acetic acid
Lysis Buffer	50 mM Tris (pH7.2), 500 mM NaCl, 10mM MgCl, 0.5% sodium deoxycholate, 1% Triton X-100, PI cocktail, Phosphatase inhibitors: 50 mM sodium fluoride and 1 µM calyculin
PBS-Tween (1x)	10 tablets of Phosphate buffered saline, 0.1% Tween-20
Running Buffer (10x)	0.25 M Tris base, 1.92 M glycine, 1% SDS
SDS Sample Loading Buffer (2x)	60mM Tris-HCl (pH 6.8), 25% Glycerol, 2.5% SDS, 0.02% Bromophenol blue
TBS-Tween (10x)	20 mM Tris-base (pH 7.5), 150 mM NaCl, 0.1% Tween-20
Transfer buffer (10x)	0.25 M Tris base, 1.86 M glycine, 10% methanol

2.2 Antibodies

Table 2.5 Antibodies

Target	*Dilution*	*Species*	*Source*
β1 integrin	1/1000 (WB)	Rabbit	Millipore
E-cadherin	1/1000 (WB)	Mouse	Abcam
EGFR	1/1000 (WB)	Rabbit	Millipore
EGFR	1/100 (IF)	Rabbit	Cell Signalling
EGFR	1/100 (FC) 1 µg (IP)	Mouse	Santa Cruz
ILK	1/1000 (WB)	Rabbit	OriGene
Kindlin-1	1/1000 (WB), 1/200 (IF)	Mouse	Moravian Biotechnology
Kindlin-2	1/1000 (WB)	Mouse	OriGene
Lamp-1	1/100 (IF)	Mouse	Developmental studies hybridoma bank
Talin clone 8D4	1/200 (WB), 1/500 (IF)	Mouse	Sigma Aldrich
Talin	1/1000 (WB)	Mouse	Millipore
Thrombospondin	1/500 (WB)	Mouse	Abcam
Zo-2	1/1000 (WB)	Rabbit	Santa Cruz
Anti-Mouse HRP	1/1000 (WB)	Goat	DAKO
Anti-Rabbit HRP	1/1000 (WB)	Goat	DAKO
Anti-mouse Alexa 488	1/400 (IF)	Goat	Molecular Probes
Anti-mouse Alexa 568	1/500 (IF)	Goat	Molecular Probes
Anti-Rabbit Alexa 488	1/400 (IF)	Goat	Molecular Probes
Anti-Rabbit Alexa 568	1/500 (IF)	Goat	Molecular Probes
Phalloidin 633	1/100 (IF)		Invitrogen

Abbreviations: Western blotting (WB), immunoflourescence (IF), flow cytometry (FC) and immunoprecipitation (IP).

2.3 Methods

2.3.1 Molecular biology and cloning

2.3.1.1 Generation of mutant constructs of kindlin-1

The following amino acid sequences are of the wild type and mutant constructs of kindlin-1. The wild type kindlin-1 amino acid sequence is 677 amino acids (GenBank accession number: NP_060141.3). The GST-kindlin-1 construct was generously given by Professor David Calderwood, Yale University, USA.

```
Wild-type:
1       MLSSTDFTFA SWELVVRVDH PNEEQQKDVT LRVSGDLHVG GVMLKLVEQI NISQDWSDFA
61      LWWEQKHCWL LKTHWTLDKY GVQADAKLLF TPQHKMLRLR LPNLKMVRLR VSFSAVVFKA
121     VSDICKILNI RRSEELSLLK PSGDYFKKKK KKDKNNKEPI IEDILNLESS PTASGSSVSP
181     GLYSKTMTPI YDPINGTPAS STMTWFSDSP LTEQNCSILA FSQPPQSPEA LADMYQPRSL
241     VDKAKLNAGW LDSSRSLMEQ GIQEDEQLLL RFKYYSFFDL NPKYDAVRIN QLYEQARWAI
301     LLEEIDCTEE EMLIFAALQY HISKLSLSAE TQDFAGESEV DEIEAALSNL EVTLEGGKAD
361     SLLEDITDIP KLADNLKLFR PKKLLPKAFK QYWFIFKDTS IAYFKNKELE QGEPLEKLNL
421     RGCEVVPDVN VAGRKFGIKL LIPVADGMNE MYLRCDHENQ YAQWMAACML ASKGKTMADS
481     SYQPEVLNIL SFLRMKNRNS ASQVASSLEN MDMNPECFVS PRCAKRHKSK QLAARILEAH
541     QNVAQMPLVE AKLRFIQAWQ SLPEFGLTYY LVRFKGSKKD DILGVSYNRL IKIDAATGIP
601     VTTWRFTNIK QWNVNWETRQ VVIEFDQNVF TAFTCLSADC KIVHEYIGGY IFLSTRSKDQ
661     NETLDEDLFH KLTGGQD
```

The 9 base pair deletion is an in-frame mutation for which homozygous individuals have a Kindler syndrome phenotype indistinguishable from other Kindler syndrome patients. The 3 amino acids deleted are from 623 to 625.

```
F3 9bp deletion:
1       MLSSTDFTFA SWELVVRVDH PNEEQQKDVT LRVSGDLHVG GVMLKLVEQI NISQDWSDFA
61      LWWEQKHCWL LKTHWTLDKY GVQADAKLLF TPQHKMLRLR LPNLKMVRLR VSFSAVVFKA
121     VSDICKILNI RRSEELSLLK PSGDYFKKKK KKDKNNKEPI IEDILNLESS PTASGSSVSP
181     GLYSKTMTPI YDPINGTPAS STMTWFSDSP LTEQNCSILA FSQPPQSPEA LADMYQPRSL
241     VDKAKLNAGW LDSSRSLMEQ GIQEDEQLLL RFKYYSFFDL NPKYDAVRIN QLYEQARWAI
301     LLEEIDCTEE EMLIFAALQY HISKLSLSAE TQDFAGESEV DEIEAALSNL EVTLEGGKAD
361     SLLEDITDIP KLADNLKLFR PKKLLPKAFK QYWFIFKDTS IAYFKNKELE QGEPLEKLNL
421     RGCEVVPDVN VAGRKFGIKL LIPVADGMNE MYLRCDHENQ YAQWMAACML ASKGKTMADS
481     SYQPEVLNIL SFLRMKNRNS ASQVASSLEN MDMNPECFVS PRCAKRHKSK QLAARILEAH
541     QNVAQMPLVE AKLRFIQAWQ SLPEFGLTYY LVRFKGSKKD DILGVSYNRL IKIDAATGIP
601     VTTWRFTNIK QWNVNWETRQ VVIEFDQNVF TAFTCLSADC KIVHEYIGGY IFLSTRSKDQ
661     NETLDEDLFH KLTGGQD
```

The W mutant is a point mutation of tryptophan at position 612 to alanine.

```
PH+F3 domain deletion:
1       MLSSTDFTFA SWELVVRVDH PNEEQQKDVT LRVSGDLHVG GVMLKLVEQI NISQDWSDFA
61      LWWEQKHCWL LKTHWTLDKY GVQADAKLLF TPQHKMLRLR LPNLKMVRLR VSFSAVVFKA
121     VSDICKILNI RRSEELSLLK PSGDYFKKKK KKDKNNKEPI IEDILNLESS PTASGSSVSP
181     GLYSKTMTPI YDPINGTPAS STMTWFSDSP LTEQNCSILA FSQPPQSPEA LADMYQPRSL
241     VDKAKLNAGW LDSSRSLMEQ GIQEDEQLLL RFKYYSFFDL NPKYDAVRIN QLYEQARWAI
301     LLEEIDCTEE EMLIFAALQY HISKLSLSAE TQDFAGESEV DEIEAALSNL EVTLEGGKAD
361     SLLEDITDIP KLADNLKLFR PKKLLPKAFK QYWFIFKDTS IAYFKNKELE QGEPLEKLNL
421     RGCEVVPDVN VAGRKFGIKL LIPVADGMNE MYLRCDHENQ YAQWMAACML ASKGKTMADS
481     SYQPEVLNIL SFLRMKNRNS ASQVASSLEN MDMNPECFVS PRCAKRHKSK QLAARILEAH
541     QNVAQMPLVE AKLRFIQAWQ SLPEFGLTYY LVRFKGSKKD DILGVSYNRL IKIDAATGIP
601     VTTWRFTNIK QWNVNWETRQ VVIEFDQNVF TAFTCLSADC KIVHEYIGGY IFLSTRSKDQ
661     NETLDEDLFH KLTGGQD
```

2.3.1.2 Site-directed mutagenesis

To create the 9 base pair deletion of kindin-1 the quickchange site-directed mutagenesis kit was used (Stratagene). The sample reaction was prepared using 5 µl of 10X reaction buffer, 1 µl (40 ng) of dsDNA template, 0.5 µl (125 ng) of oligonucleotide primer forward, 0.5 µl (125 ng) of oligonucleotide primer reverse, 1 µl of dNTP mix and double distilled water to a final volume of 50 µl. Then 1 µl of *PfuTurbo* DNA polymerase (2.5 U/µl) was added. Each PCR cycle consisted of an initial denaturation (95 °C, 30 seconds) for 1 cycle, denaturation (95 °C, 10 seconds), annealing (gradient temperature 55-70 °C), extension (68 °C, 1 min/kb) for 18 cycles. Following temperature cycling, the reaction was placed on ice for 2 mins to cool the reaction to ≤37 °C. 1 µl of the *Dpn*I restriction enzyme (10 U/µl) was added directly to the amplification reaction and spun in a microcentrifuge for 1 min. The reaction was incubated at 37 °C for 1 hr to digest the parental (i.e., the nonmutated) supercoiled double stranded DNA. 1 µl of the solution was then transformed into XL1-blue supercompetent cells (Stratagene).

2.3.1.3 Primers for generating mutants

The following primers were designed to generate the site-directed mutagenesis of kindlin-1.

Kindlin-1 9 bp deletion forward

5’ AACCCGGCAGGTGGTTGACCAAAACGTCTT 3’

Kindlin-1 9 bp deletion reverse

5’ TTGGGCCGTCCACCAACTGGTTTTGCAGTT 3’

2.3.1.4 Transformation of pGEX GST vectors

2 µl of DNA was added into 25 µl of BL21 competent cells, left on ice for 30 min and heat shocked at 42ºC in a waterbath for 45 seconds and then left in ice for 2 min. 250 µl SOC media was added and placed in a 37 ºC shaking incubator for 1 hr. Finally the solution was streaked on an agar plate and left in the 37 ºC incubator overnight for growth.

2.3.1.5 Bacterial Transformation

Transformations were performed according to protocols provided by Invitrogen. Briefly, One Shot Top10 E.coli were thawed on ice, then 1-5 µl DNA was added to each vial (50 µl) of competent cells and were incubated for 30 mins on ice. This was followed by incubation at 42 ºC for 45 seconds and then immediate incubation on ice for 2 mins. 250 µl S.O.C. media was then added and incubated for 1-2 hrs shaking at 37 ºC. LB agar plates containing Kanamycin were then spread with the solution. Plates were inverted and incubated at 37ºC overnight.

2.3.1.6 Restriction digests

Restriction digests were performed according to protocols provided by New England BioLabs. Briefly, the restriction digest was made up of 2-3 µg DNA, the appropriate

NEbuffer (1X) (New England BioLabs), BSA (1X) (New England BioLabs), 1 µl restriction enzyme (New England BioLabs) per 1 µl DNA and made up to 50 µl with distilled water. For double digest both enzymes were added at the same time, or if sequential the second restriction enzyme was added after digest with the first restriction enzyme. Loading buffer was added to each digest and they were run on a 1% agarose gel containing 1:50 (1% stock, 0.02% final) ethidium bromide.

2.3.1.7 Gel extraction

DNA was extracted using the QIAquick gel extraction kit according to the protocol provided by QIAGEN. Briefly, the DNA fragment was removed from the agarose gel and 3 volumes of Buffer QG were added to 1 volume of gel. The gel was then incubated at 50 ºC for 10 mins until the gel had dissolved and 1 gel volume of isopropanol was added to the sample and mixed. The sample was placed in a QIAquick spin column and was centrifuged for 1 min. The sample was washed with 500 µl of Buffer QG and centrifuged for a further 1 min, and also washed with 750 µl of Buffer PE and centrifuged for a further 1 min. The column was centrifuged for a further 1 min and placed in a clean 1.5 ml microcentrifuge tube, 30 µl elution buffer was added to the column and left to stand for 1 min. Then the column was centrifuged for 1 min to elute the DNA. A nanodrop spectrophotometer was used to quantify the concentration and purity of the DNA.

2.3.1.8 Minipreps

1 ml of bacterial overnight culture was centrifuged at 13,000 rpm for 5 min. The supernatant was discarded and the pelleted bacterial cells were resuspended in 250 µl of Buffer P1. Before using Buffer P1, LyseBlue reagent was added to Buffer P1 at a

ratio of 1 to 1000, RNase was also added and mixed in. 250 µl of Buffer P2 was then added and mixed thoroughly by inverting the tube 4–6 times until the solution became clear. The solution turned blue due to the addition of LyseBlue reagent. 350 µl of Buffer N3 was added and mixed immediately and thoroughly by inverting the tube 4–6 times or until the solution became colourless. The solution was centrifuged for 10 min at 13,000 rpm (~17,900 x *g*) in a table-top microcentrifuge at room temperature. The supernatant was transferred to the QIAprep spin column and centrifuged for 1 min and the flow-through was discarded. To wash the QIAprep spin column, 0.75 ml Buffer PE was added and centrifuged for 1 min and the flow-through was discarded. The QIAprep spin column was centrifuged again for 1 min to remove residual wash buffer. To elute DNA, 50 µl of water was added to the centre of the QIAprep spin column, and allowed to stand for 1 min at room temperature and finally centrifuge for 1 min. The flow-through contained the DNA which was measured using a nanodrop spectrophotometer (Labtech international).

2.3.1.9 Midipreps

25-50 ml of bacterial culture was grown up overnight and pelleted by centrifugation at 13000 rpm for 15 min. The pellet was resuspended by pipetting in 2 ml of Buffer P1. 2 ml of Buffer P2 was added and gently mixed by inverting and incubated at room temperature for 3 min. Due to addition of LyseBlue reagent to Buffer P1, the cell suspension turned blue after addition of Buffer P2. Then a further 2 ml of Buffer S3 was added to the lysate and mixed immediately by inverting 4–6 times. Addition of Buffer S3 caused a fluffy white precipitate to form containing genomic DNA, proteins, cell debris, and SDS. The suspension was mixed until all trace of blue was gone and the suspension was colourless. The mixture was transferred into the

QIAfilter cartridge and incubated at room temperature for 10 min. A plunger was inserted into the QIAfilter cartridge and the cell lysate was filtered the into a new tube, to which 2 ml of Buffer BB was added and mixed by inverting 4–6 times. The lysate was then transferred to a QIAGEN Plasmid Plus Midi spin column with a tube extender attached on the QIAvac 24 Plus. Using vacuum, the solution was drawn through the column. DNA bound to the column was washed with 0.75 ml of Buffer and again washed with 0.7 ml of Buffer PE. To completely remove the residual wash buffer, the column was centrifuged for 1 min at 10,000 x g in a microcentrifuge. The column was then placed into a clean 1.5 ml microcentrifuge tube and to elute the DNA, 200 µl of water was added to the centre of the column, with 1 min incubation at room temperature, and then finally centrifuged for 1 min.

2.3.2 Cell culture

2.3.2.1 Cell lines and Immortalisation of keratinocytes by human papilloma virus (HPV)

Frozen vials of keratinocytes from two patients with KS harbouring known *KIND1* mutations (c.676insC/c.676insC and p.Glu304X/p.Leu302X) were immortalised by Dr Andrew South, University of Dundee, Scotland. Immortalisation of keratinocytes was performed by infecting the cells with the human papilloma virus, 208 HPV16 E6 and E7. The ability of immortalizing human keratinocytes has previously been localized to the E6 and E7 region of HPV16 viral DNA (Halbert *et al.*, 1991). The immortalised keratinocytes were then grown in serum-free keratinocyte growth medium. Their subsequent maintenance and passage were similar to those of primary human keratinocytes. Age- and gender-matched NHK were immortalised using an identical protocol. All experiments were performed between 15 and 25 passages after

immortalisation. All patient cells were obtained under a St Thomas' Hospital Ethics Committee approved project – Molecular basis of inherited skin disease - 07/H0802/104. Keratinocyte SFM media (Gibco), supplemented with EGF and bovine pituitary extract was used to grow and maintain cells. The cells were thawed out in this media and transferred to a T25 cm^2 flask, and placed in a 37 °C incubator to allow cells to attach and grow. To passage the cells, they were washed once with PBS (without Ca and Mg, APP) and trypsinised (Trypsin in EDTA at 0.05% concentration). After detachment the cells were centrifuged for 3 min at 1200 rpm and resuspended in 10 ml SFM media which was divided between two flasks.

2.3.2.2 Producing lentiviral particles and infecting cells

To generate stable cell lines of normal and KS keratinocytes overexpressing wild type kindlin-1 or the tryptophan mutant form of kindlin-1, both of these were cloned into mCherry lentivector backbones. The original pHR9SIN-SEW lentiviral expression vector was a gift from Dr Adrian Thrasher (Institute of Child Health, UCL, London), and the mCherry protein tag was subsequently cloned into this vector.

For each plasmid to be transfected, a flask of T75 cm^2 of HEK293T was plated. The transfection mix was prepared with a total of 5 µg of DNA (2.5 µg target plasmid .i.e. WT mCherry-kindlin-1 or mutant mCherry-kindlin-1-W612A, 1.9 µg of packaging plasmid Δ8.91 and 0.6 µg of envelope plasmid pMD2.G in 50 µl of OPTI-MEM. A mastermix of FuGENE transfection reagent in serum-free OPTI-MEM was also created. This consisted of 6 µl of FuGENE and 74 µl of OPTI-MEM for every reaction. These were left to incubate for 5 min at room temperature. The FuGENE mastermix was then added to the DNA transfection mix and incubated for a further

20 min at room temperature. The DNA:FuGENE mix was added to the HEK293T cells, the media in these cells were changed to OPTI-MEM beforehand to remove the presence of antibiotics. The cells were incubated for overnight at 37 ºC. The media was then changed to remove the transfection reagent and replaced with DMEM plus 10% FBS and penicillin/streptomycin and again left to incubate for 24 hrs at 37 ºC. At this point the virus was harvested by removing the media, which contained the virus particles and centrifuging at 1200 rpm for 3 min as well as filtering through a 0.45 µm filter to remove cells. The cells were replaced with fresh media and the virus was collected again after 24 hrs. The virus was used immediately for infecting cells or was stored at -80 ºC.

For infection of the target cells, normal and KS keratinocytes were plated on 6 well plates and grown to 70% confluency. The media was replaced but with the addition of polybrene at a concentration of 8 µg/ml to increase efficiency of viral infection. 1 ml of lentiviral particle solution was added to the cells and left to incubate at 37 ºC. The media was changed after 24 hrs to remove the virus and cells were grown and passaged. A fluorescence microscope was used to check expression of mCherry tagged protein in the keratinocytes. To obtain a pure population of stably expressing mCherry tagged kindlin-1 proteins, the cells were put through fluorescence activated cell sorting (FACS). The cells were trypsinised and centrifuged at 1200 rpm for 3 min. They were then resuspended in 1 ml of SFM media with 4% FBS. Only cells that were mCherry-positive were collected and plated on T25 cm^2 flasks and used for further experiments.

2.3.3 Protein production, purification and biochemical analysis

2.3.3.1 GST-kindlin protein purification

DNA encoding GST-kindlin-1 proteins were transformed into BL21 cells for optimal expression/purification. Colonies were added to 30 ml LB with Ampicillin at a concentration of 100 µg/ml and left to grow overnight in a shaking incubator at 37 °C, 250 rpm. Cultures were seeded evenly into 6 x 1 litre flasks (800 ml LB) containing ampicillin (100 µg/ml) and left to grow at 37°C until optical density (OD^{600}) reached approximately 0.4 and then for a further 30 min at 16 °C. IPTG (1:1000) was added to the bacterial culture which was left in the shaking incubator overnight at 16 °C, 225 rpm. Bacteria were pelleted and resuspended in 25 ml per litre of lysis Buffer: 50 mM phosphate, 300 mM NaCl, 5 mM imidazole, pH 8.0. This was then transferred to 50 ml Falcon tube and placed at -80 °C overnight to freeze. The cells were thawed at room temperature in a water bath with mixing. 500 µl of lysozyme (20 mg/ml of water) was added and rocked for 15 min at room temperature, and left to stand at room temperature for 15 min. Cells were sonicated on ice (20 pulses of 10 seconds (200 seconds total) at power 10 with 10 seconds of cooling in between). After sonication, 50 µl of *DNase*I per litre centrifuge tubes was added and placed in a rocker for 15 min. The cell debris was pelleted by centrifugation. 0.5 ml of Glutathione sepharose beads was added to 5 ml of lysis buffer (for washing) and spun at 1000 rpm for 5 min at room temperature and aspirated. The pre-cleared lysate was poured into 50 ml Falcon tubes containing beads and was allowed to mix overnight at 4 ºC. Finally the beads were washed with lysis buffer 3 times and resuspended in 1 ml lysis buffer with protease inhibitor.

2.3.3.2 GST pulldowns

Keratinocytes were washed twice with ice-cold PBS. Cold lysis buffer containing protease inhibitor, sodium fluoride and calyculin was added. The keratinocytes were scraped into lysis buffer (50 mM Tris (pH7.2), 500 mM NaCl, 10 mM MgCl, 0.5% sodium deoxycholate, 0.5% Triton X-100, PI cocktail, phosphatase inhibitors: 50 mM sodium fluoride and 1 µM okadaic acid), 500 µl for ~80% confluent T75 cm^2 of cells) and transferred into 1.5 ml tubes. This was left on a rotator at 4 ºC for 30 mins to ensure complete lysis and then centrifuged at 13,000 rpm for 1 min at 4 ºC to pellet insoluble material. 20 µl of the sample of cell lysate was removed before adding lysate to beads to allow analysis of total protein on the same gel as the pulldown samples. The cleared lysates were transferred into tubes with 50 µl GST-tagged protein and placed on a rotator at 4 ºC for 60 mins. The beads were washed 4 times with ice-cold lysis buffer. After the final wash, the wash buffer was removed and SDS sample buffer was added. The sample was boiled for 10 mins and run on a 10% acrylamide gel.

2.3.3.3 SDS PAGE analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) separates protein based on molecular weights. SDS loading buffer was added to the samples which denatured the protein from its tertiary structure to the basic amino acid primary structure. The SDS also induced a negative charge to proteins so that when an electrical charge was applied the protein would migrate towards the cathode pole. Gels of 8% or 12 % were made in 1.5 mm cassettes with stacking gel on top and resolving gel beneath. Along with the samples, a marker was run which includes molecular weight standards of known sizes. The gel was run for 1.5 hrs at 100 V.

2.3.3.4 Silver staining

The polyacrylamide gels were silver-stained using the plusone silver staining kit (GE Healthcare) to provide higher sensitivity for band detection. The gel was first left in fixing solution 40 % ethanol for 30 min (Ethanol 100 ml, glacial acetic acid 25 ml, water to 250 ml) and then left in sensitising solution for 30 min (ethanol 75 ml, sodium thiosulphate (5 % w/v) 10 ml, sodium acetate 17 g). The gel was washed with water 3 times for 5 min and silver solution was added (silver nitrate solution (2.5 % w/v) 25 ml, water to 250 ml). After 20 min, the gel was washed 2 times for 1 min and then developing solution was added (sodium carbonate 6.25 g, water to 250 ml, 0.2 ml formaldehyde 37 % w/v), the gel was transferred to stopping solution when bands reached the desired intensity (EDTA-$Na_2 \cdot 2H_2O$ 3.65 g, water to 250 ml). After washing for a further 3 times for 5 min the gel was left in preserving solution (ethanol 75 ml, glycerol 11.5 ml, water to 250 ml) for 30 min, twice.

2.3.3.5 Western Blotting

Samples for western blotting were prepared from cell lysates and/or pulldown experiments. The protein samples were loaded onto a Tris-Glycine polyacrylamide gel alongside a protein ladder and run for 1.5 hours at 100 V. The proteins were transferred onto nitrocellulose membranes (Amersham Biosciences) for 1 hr at 100 V using a transfer kit (Biorad). The membranes were blocked using % Milk/PBS or 3% BSA/TBS for 1 hr at room temperature. The membranes were then probed with the primary antibody overnight at 4 ºC or for 3 hrs at room temperature. The membranes were then washed 3 times for 10 mins each with PBS-Tween (0.1% Tween in PBS) prior to incubation with horseradish peroxidase-conjugated secondary antibodies for 1 hr at room temperature. Membranes were then washed a

further 3 times and proteins were detected by ECL chemiluminescence kit (Thermo Scientific), exposed on Medical X-ray film (Fuji) and developed using a Xograph compact X4 developer (Imaging Systems). For reprobing, blots were stripped with Re-blot strong (10X) (Chemicon) diluted to 1X in distilled water for 10 mins at room temperature, and then treated as before.

2.3.3.6 Immunoprecipitation (IP)

Cells were cultured until 80% confluent in T75 cm^2 tissue culture flasks and lysed in 500 µl IP buffer on ice. A/G agarose affinity matrix suspension was washed 3 times with PBS prior to use. Lysates were pre-cleared with 100 µl washed A/G agarose affinity matrix suspension rotating at 4 ºC for 30 mins, and 50 µl lysate was kept (1:1 IP buffer: sample buffer) prior to pre-clearing at -20 ºC for an input sample. Pre-cleared lysates were centrifuged and the supernatants were collected. Each IP experiment had a duplicate of primary antibody and control IgG. Antibody or control IgG was incubated with 200 µl pre-cleared lysate tumbling at 4 ºC overnight. Then 50 µl of washed A/G agarose affinity matrix suspension was added to the antibody lysate mix for 2 hrs tumbling at 4 ºC. Alternatively, antibody was pre-incubated with beads overnight tumbling at 4 ºC. Then, excess antibody was removed by washing 3 times with PBS, and pre-cleared lysate was added to each antibody A/G agarose affinity matrix suspension mix for 2 hrs tumbling at 4 ºC. After antibody, lysate and A/G agarose affinity matrix suspension incubation, the mixtures were centrifuged and 25 µl supernatant (1:1 supernatant: sample buffer) was kept for an unbound sample and was stored at -20 ºC. The rest of the supernatant was removed and the A/G agarose affinity matrix suspension was washed three times for 10 mins in IP buffer. Samples were either used immediately or stored at -20ºC (1:1 IP: reducing

sample buffer). Lysates were boiled at 95 ºC and centrifuged to clear cell debris before use. 40 µl of each sample was loaded in each well of 8% SDS-PAGE gels in 1.5 mm cassettes and subjected to SDS-PAGE, and then the Western blotting protocol was followed. Each IP was performed at least 3 times.

2.3.3.7 Mass spectrometry

The bands cut out from pulldown analysis gels were sent off for mass spectrometry analysis to Aberdeen Proteomics (University of Aberdeen, School Medical Sciences, Lab 1.35, IMS Building, Foresterhill, Aberdeen). The samples were first digested with trypsin to obtain smaller samples which were then ionised. The ions were accelerated and deflected by a magnetic field. The analyser region of the mass spectrometer separated the ions according to their mass (m) to charge (z) ratios (m/z). The separated ions were then detected and the signal was recorded. The mass to charge ratio was recorded along with the relative abundance to be presented on a m/z spectrum. The experiment was performed in a vacuum to allow the ions to move freely without colliding into air molecules. Data were analysed using MASCOT search software and databases to determine the identity of the bands.

2.3.4 Flow cytometry (FC)

Confluent T25 cm^2 flask of cells were scraped in 5 ml of PBS and centrifuged at 1200 rpm for 3 mins followed by fixation in 500 µl 4% PFA/PBS for 30 mins on ice. Cells were then washed 3 times in 500 µl of PBS and blocked in 100 µl 5% BSA/PBS for 30 mins on ice. Cells were then incubated with primary antibody in 100 µl 5% BSA/PBS for 2 hrs at 4 ºC. Samples were divided to include a secondary only negative control, which was not incubated with any primary antibodies. The

samples were washed 3 times in 500 µl PBS and incubated with secondary antibody in 100 µl 5% BSA/PBS for 30 min. They were then washed a further 3 times in 500 µl PBS and finally re-suspended in 500 µl of PBS. Cells were analysed on a BD FACSCalibur flow cytometer (BD Biosciences) with CellQuest Pro software. Gates were set to exclude outlying cells and then histograms were plotted to show cell count against intensity of staining for FITC. For FITC intensity, the mean value for cells only incubated with secondary antibody was deducted from the mean values for the other samples to compensate for non-specific intensity values. Gates were set to count positive FITC cells, giving a measure of the amount of positive FITC cells for each cell type. Each experiment was repeated at least 3 times and the corrected mean values were averaged and plotted as histograms using Excel.

2.3.5 Microscopy

2.3.5.1 Immunofluorescence

The cells were plated onto coverslips on 4 well plates. Coverslips were coated with human plasma fibronectin (1/10) (Millipore) for a minimum of 30 mins and then washed once with PBS. The cells were plated in SFM media and allowed to grow overnight at 37 °C. At this point, cells were fixed using 4% para-formaldehyde (PFA/PBS) for 10 mins, washed twice with PBS and then permeabilised with 0.2% Triton-X/PBS for 10 mins. Coverslips were again washed with PBS and blocked with 1% BSA/PBS for 20 mins. The selected primary and secondary antibodies were diluted in 3% BSA/PBS for 3 hrs and then 2 hrs at room temperature successively. Secondary antibodies (Alexa Fluor 488 anti-mouse, Alexa Flour 568 anti-rabbit and Alexa Fluor 633 phalloidin; Invitrogen). Coverslips were washed with PBS and distilled water and finally mounted on slides using mounting media (FluorSave

reagent, Calbiochem). Images of fixed cells were acquired on a Zeiss LSM 510 confocal microscope (Carl Zeiss Inc., Oberkochen, Germany).

2.3.5.2 Immunohistochemistry

5 μm thick frozen skin sections were cut from skin biopsies obtained from control and patients with KS in whom loss-of-function *KIND1* mutations had been determined. Skin biopsy samples were previously embedded vertically in Tissue-Tek OCT Compound (VWR International, Lutterworth, Leicestershire, UK) and frozen in liquid nitrogen-cooled isopentane. The blocks were then stored at -80 °C for later use. Prior to cryosectioning, the tissue blocks were equilibrated to -20 °C in the cryostat. The skin sections were air-dried for at least 1 hr. The sections were fixed with 4% PFA for 10 min and permeabilised with 0.2% Triton-X for 5 min. Each section was then blocked for 1 hr at 37ºC using 10% FBS in PBS and then washed in PBS. The sections were then incubated with the relevant primary antibody diluted in 3% BSA/PBS solution overnight at 37 °C. The sections were then washed three times with PBS before being incubated with secondary antibody and DAPI diluted in 3% BSA/PBS solution for 1 hr at 37ºC. Following a further PBS wash, the sections were mounted by adding a drop of mounting media and placing a coverslip on top. Stained sections were viewed under a Zeiss LSM 510 confocal microscope (Carl Zeiss Inc.)

2.3.5.3 Random migration assay

For the random migration assay, NHK, KS, WT kindlin-1 and mutant kindlin-1-W612A overexpressing KS cells were seeded on 12 well plates. The cells were starved overnight by replacing the media with optimem. The cells were stimulated

with EGF at a concentration of 100 ng/ml. The cells were imaged with time-lapse microscopy before and after stimulation. Live cell imaging was performed on Zeiss Axiovert 100 microscope using an automated scanning stage. Images were acquired by phase contrast imaging using a 10x N-Achroplan Phase contrast objective (numerical aperture 0.25). Cell images were collected using a Sensicam (PCO Cook) charge coupled device (CCD) camera, taking a frame every 10 mins for 16 hrs using AQM acquisition software (Andor Bioimaging, Belfast, U.K.). Subsequently, all the acquired time-lapse sequences were converted to movie files (.AVI) using ImageJ and cells from the time-lapse sequence were tracked using IQ Tracking Software (Andor Bioimaging). At least 60 cells were tracked for each sample. Tracking resulted in the generation of a sequence of position coordinates relating to each cell in each frame. Motion analysis was then performed on these sequences using Wolfram Mathematica 6.

2.3.5.4 Confocal microscopy

Images of fixed cells were acquired on a Zeiss LSM 510 confocal microscope (Carl Zeiss Inc.) or Nikon A1R confocal microscope (Nikon Instruments UK). Excitation wavelengths of 488 nm (Argon laser), 543 nm (HeNe1 laser) and 633 nm (HeNe2 laser) were used. Images were taken using a 63x oil Plan Fluor immersion objective (numerical aperture 1.4). Images were exported from Database Files.mdb to TIFF files by Zeiss LSM Image Browser and processed with ImageJ software.

2.3.5.5 Image analysis

For colocalisation analysis the confocal images were processed with the ImageJ plugin:http://imagejdocu.tudor.lu/doku.php?id=plugin:analysis:jacop_2.0:just_another_colocalization_plugin:start

For analysis of surface levels vs. total levels of EGFR or kindlin proteins, images were manually thresholded in ImageJ and intensity values were calculated per cell area (corrected for total μm) and normalised to the control cells in that sample set.

2.3.5.6 Statistical analysis

All statistical tests were performed using Students T-tests (Excel) except for the random migration data which was analysed using ANOVA (SPSS). Significance was taken as $p<0.05$ and significance values were assigned in specific figures/experiments as shown.

Chapter 3

Identification of novel proteins regulated by kindlin-1 expression

3.1 Introduction

Kindlin-1 is predominantly expressed in epithelial tissue in the skin and the intestine. In the skin it is found to be highly expressed in keratinocytes of the epidermis (Siegel *et al.*, 2003). Keratinocytes containing mutations in the *KIND1* gene have been previously reported to show various abnormal phenotypes which indicate the importance of kindlin-1 regulation of normal function in these cells. The array of defects include reduced cell adhesion, spreading, migration and proliferation, as well as abnormal cell shape, loss of polarisation and multiple leading edges (Herz *et al.*, 2006; Has *et al.*, 2008). Directed migration of cells is required for normal development, and also in regenerative processes, such as wound healing. A main feature of migrating cells is the lamellipodium which consists of polarised network of actin filaments that form the protruding front of a motile cell. Migration is dependent on integrin-mediated adhesion and changes to the cytoskeleton and is controlled by Rho GTPases (Ridley, 2001). Kindlin-1 can bind and activate integrins in cooperation with talin, thus kindlin-1 deficient keratinocytes are not able to carry out these important integrin dependent processes. Kindlin-1 deficient cells have reduced active Rho GTPases and their downstream effectors (Has *et al.*, 2009), showing that there is disruption in the propagation of the downstream signalling of integrin that controls cell migration and polarisation. Integrins are heterodimeric transmembrane receptors, their primary function is mediating cell-cell and cell-matrix interactions and internal linkage to the cytoskeleton and cell signal transduction (Hynes, 2002). Integrins mediate signalling from the extracellular space into the cell via adaptor molecules such as FAK, ILK and particularly interesting new cysteine-histidine rich protein (PINCH) (Harburger and Calderwood, 2009). Activated pathways signal a variety of processes including survival, apoptosis,

proliferation, cell-cycle progression, cell shape, polarity, adhesion, migration and differentiation (Hynes, 2002). Integrins bind to cell surface and ECM components such as fibronectin, vitronectin, collagen, and laminin and cell associated ligands such as ICAMs and VCAMs (Harburger and Calderwood, 2009). Integrin ligands also include matrix metalloproteinase (MMP) and thrombospondin (TSP) (Bein and Simons, 2000; Chandrasekaran *et al.*, 2000).

TSPs are matricellular glycoprotein that mediates cell-matrix interaction. There are five members in the TSP family, which are subdivided into two groups. TSP-1 and TSP-2 are homotrimers, while TSP-3, TSP-4 and TSP-5 are homopentamers and lack the type 1 or TSP repeats responsible for the important functions of TSP-1 and TSP-2. TSP-1 and TSP-2 are the best studied although they differ significantly in their expression pattern (Adams and Lawler, 2004). In normal human skin, TSP-1 is expressed by basal epidermal keratinocytes and is deposited in the dermal-epidermal basement membrane. It helps to form a protective barrier against ingrowth of blood vessels into the dermis (Yano *et al.*, 2005). TSP-1 is involved in various biological processes, including cell proliferation, migration and differentiation of a number of cell types (Adams and Lawler, 2004). TSP-1 has multiple domains which facilitate its activities including the amino-terminal heparin-binding domain, the procollagen domain, the properdin-like type I repeats, and the globular carboxy-terminal domain. The protein also contains type II repeats with epidermal growth factor-like homology and type III repeats that contain an RGD sequence (Adams and Lawler, 2004). The different domains enable it to interact with cell-surface receptors, ECM, growth factors and proteases which allow it to positively and negatively modulate cell adhesion, growth and motility (Chen *et al.*, 2000). One of the major functions of TSP-1 is its role in angiogenesis. Angiogenesis is the physiological process

involving growth of new blood vessels and this is vital during normal growth and development as well as in wound healing and tumour development. TSP-1 also acts to prevent angiogenesis by inhibiting endothelial cell migration. This is mediated via an interaction of the type 1 repeats of TSP-1 with β1 integrin in a PI3K dependent manner (Short *et al.*, 2005). Studies have shown that downregulation of TSP-1 results in a switch to an angiogenic phenotype. One factor that can modulate an angiogenic switch is UV radiation, as TSP-1 is downregulated in UVB radiated skin (Howell *et al.*, 2004). Further studies have confirmed that UV exposure reduces TSP-1 expression in human skin and keratinocytes and that this is mediated by activation of the PI3K-Akt pathway (Yano *et al.*, 2005; Kim *et al.*, 2006). KS patients also display sensitivity to UV and accelerated skin ageing, both of which have been linked to TSP-1 expression previously. However, any link between kindlin-1 expression and TSP-1 has not been previously explored. Indeed, the role of kindlin-1 in integrin-mediated processes provides insight into some of the phenotypes of KS such as epithelial fragility; however, there is still a need for further investigations into the pathogenic mechanisms underlying the other clinical features noted in KS patients. The aim of this chapter was to screen for novel targets downstream of kindlin-1 using mass spectrometry. This analysis revealed a number of interesting new kindlin-1 dependent proteins, including TSP-1, which may be involved in the pathophysiology of this disease.

3.2 Results

3.2.1 Characterisation of normal and KS keratinocytes

To characterise the morphology of the three cell lines used in the project, the NHK and the two KS keratinocyte cell lines were plated on coverslips and phase contrast images were acquired to show the overall shape and morphology of the cells (Figure 3.1 (a)). Images demonstrated that NHK show differences in morphology compared to that seen in KS patient cells. Control NHK appeared to be rounded in shape, while KS(1) keratinocytes were more elongated and KS(2) keratinocytes were extremely rounded up. This could be as a result of inefficient speading of the cells which has previously shown by other studies (Kloeker *et al.*, 2004; Herz *et al.*, 2006). To analyse the levels of *KIND1* mRNA in the NHK and KS cells, cells were subjected to semi-quantitative reverse transcriptase PCR. This technique assesses relative levels of mRNA by generating cDNA from the mRNA and then amplifying genes of interest using specific primers. Kindlin-1 levels were assessed and *GAPDH* was used a control to ensure equal loading between samples (Figure 3.1 (b)). Data demonstrated a marked reduction of mRNA levels in both KS keratinocytes.

In addition, Immunofluorescence staining of the three cell lines with an anti-kindlin-1 antibody was performed to analyse localisation of the protein. In NHK, kindlin-1 was found at the periphery of the cells but was also present in the cytoplasm (Figure 3.2 (a)). By comparison, KS keratinocytes showed a distinct lack of kindlin-1 staining at the cell periphery but there was still signal present in the cytoplasm (Figure 3.2 (a)). Immunohistochemistry was also performed on normal human skin (NHS) and KS patient skin (Figure 3.2 (b)). In NHS, kindlin-1 is predominantly expressed in the basal keratinocyte layer close to the dermal-epidermal junction. However, in KS skin there was a marked absence of kindlin-1 expression. Moreover,

in KS skin there was a striking loss of rete ridges giving rise to a flat appearance to the dermal-epidermal junction, as well as an atrophic epidermis, and overlying hyperkeratosis. A secondary only control shows some non specific staining on normal skin.

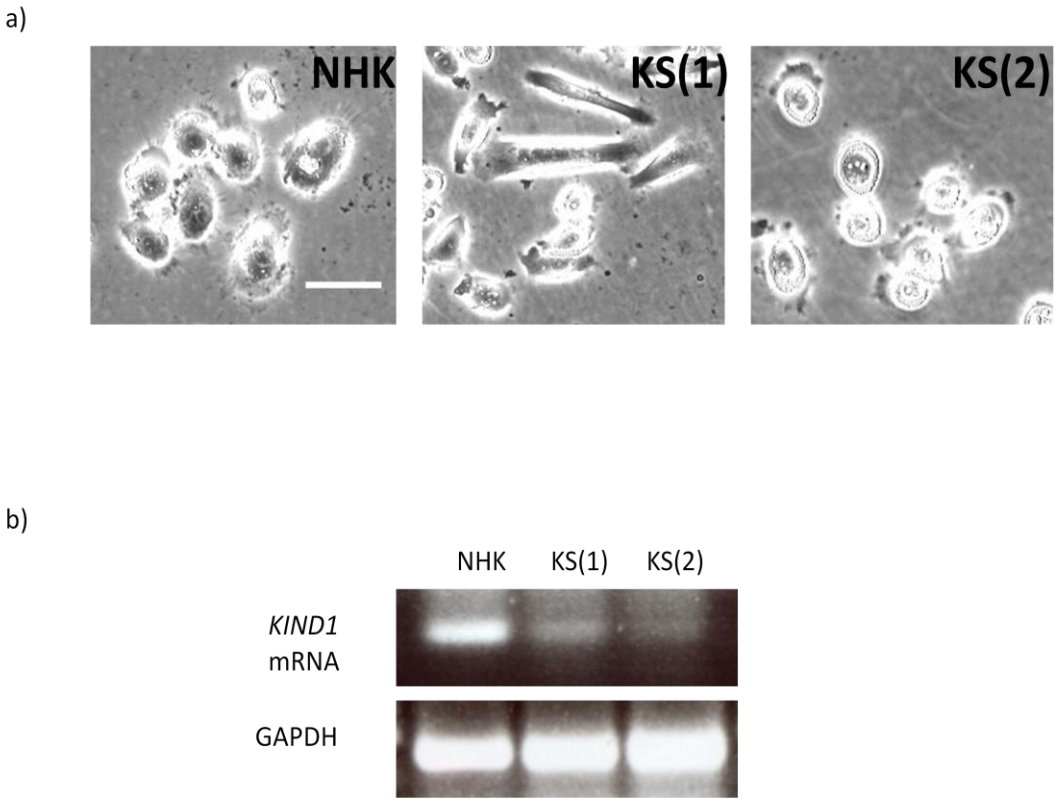


Figure 3.1: Characterisation of NHK and KS keratinocytes.

Characterisation of NHK and KS patient keratinocytes. a) Phase contrast images of the three cell lines; normal human keratinocytes (NHK), KS keratinocytes (KS(1) and KS(2)). Scale bar = 50 μm. b) Semi-quantitative RT-PCR of *KIND1* mRNA levels in NHK and KS keratinocytes. *GAPDH* serves as a loading control.

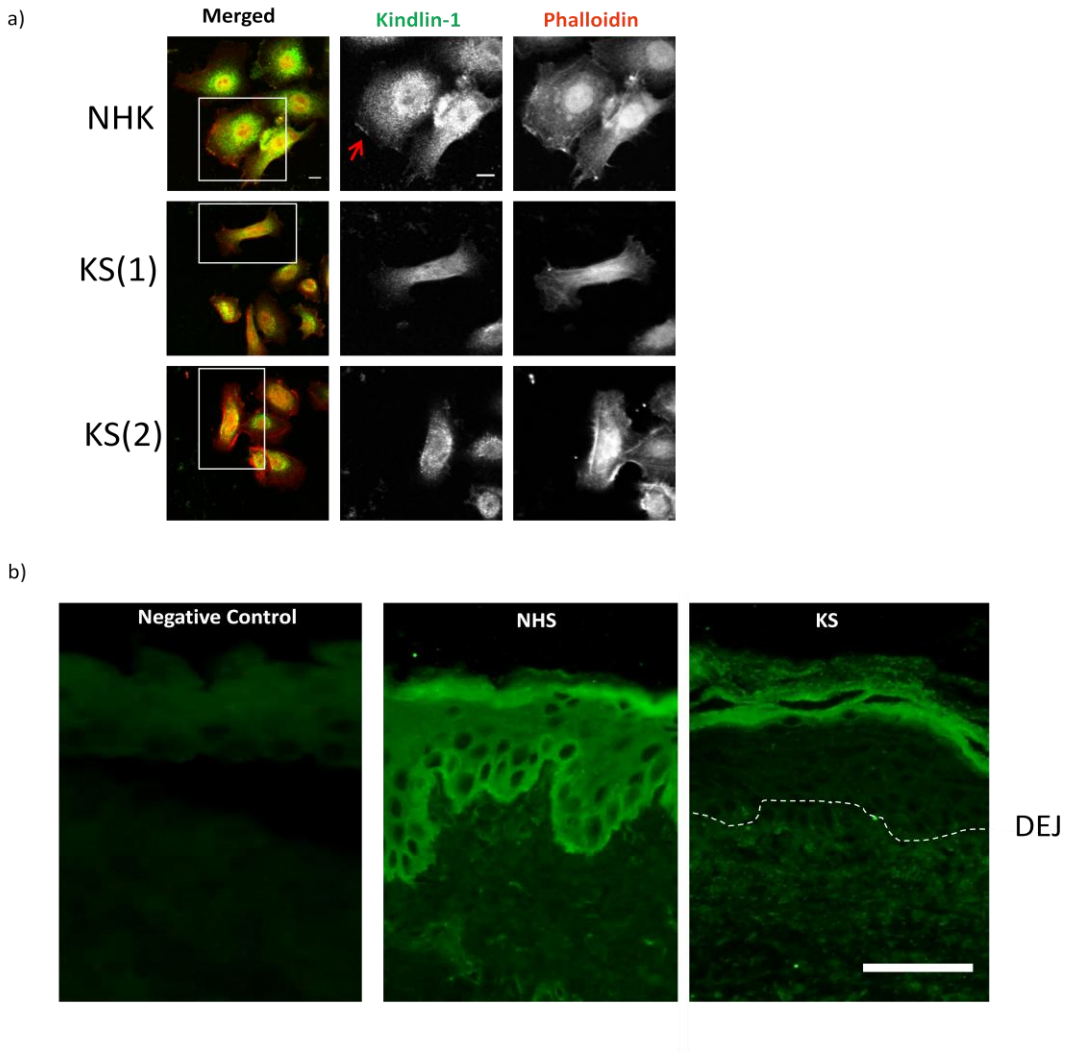


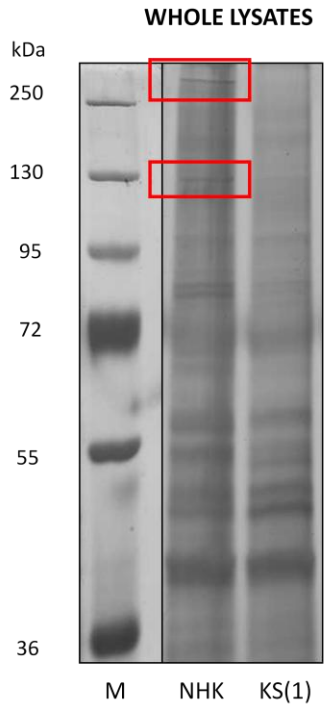
Figure 3.2: Localisation of kindlin-1.

Characterisation of kindlin-1 in NHK and KS patient keratinocytes and skin. a) Confocal images of the localisation of kindlin-1 in NHK and KS keratinocytes. Endogenous kindlin-1 is shown in green and F-actin in red (Phalloidin 633) Kindlin-1 and phalloidin channels have been zoomed in from the original image as highlighted by the white boxes. Red arrow indicates localisation of kindlin-1 at the cell periphery. Scale bar = 10 μm. b) Confocal images of the localisation of kindlin-1 in normal human (NHS) and KS patient skin (KS) with secondary only conrol. Dashed line indicates dermal-epidermal junction (DEJ). Scale bar = 50 μm.

3.2.2 Mass spectrometry analysis of proteins expressed in KS cells

To determine whether there were any other differences in protein expression between NHK and KS keratinocytes, the cells were grown to confluency and lysed. The lysates were run on a SDS-PAGE gel and stained with silver staining solutions. This allowed higher sensitivity of the detection of protein bands and allowed the band profiles of the lysates to be compared and assessed for any differences. The bands that were consistently seen to be downregulated or upregulated in at least three different experiments were cut out and subjected to mass spectrometry analysis (by Aberdeen Proteomics, University of Aberdeen). Examples of some of the bands that were excised and analysed are highlighted in Figure 3.3 (a) by white boxes.

a)



b)

Reduced protein expression in KS keratinocytes	Size of band cut from gel
Keratin 14	130 kDa
Diaphanous 1 (mDia1)	130 kDa
Plectin	Above 250 kDa
ROCK1	130 kDa
Filamin A	130 kDa
Zona Occludens 2 (ZO-2)	130 kDa
E-Cadherin	95 kDa
Epidermal growth factor receptor (EGFR)	130 kDa
Thrombospondin-1 (TSP-1)	130 kDa

Figure 3.3: Different protein banding patterns seen in NHK and KS lysates.

SDS-PAGE analysis of NHK and KS(1) lysates. a) Silver stained 12% acrylamide gel comparing lysates from NHK and KS lysates. Red boxes highlight examples of differentially expressed bands that were cut out for mass spectrometry analysis. The band was cut only from the NHK lysate sample and not from KS. b) Table of results from mass spectrometry analysis of proteins downregulated in KS keratinocytes, also showing the size of the protein bands from which they were identified.

The proteins that were identified in the bands were sequenced and the results of the mass spectrometry analysis from multiple samples are listed in Figure 3.3 (b). Immunoblotting was then used to validate the results from the mass spectrometry data. Keratin 14, mDia1, plectin, ROCK 1, filamin A, ZO-2, E-cadherin, EGFR and TSP-1 were assessed using immunoblotting followed by quantification with densitometry analysis from at least 3 independent experiments as shown in Figure 3.4 (a) and (b). The total protein levels of keratin 14, mDia1, plectin, ROCK1, filamin A and ZO-2 were not significantly different between NHK vs. KS keratinocytes. However, E-cadherin, EGFR and TSP-1 levels were significantly reduced in both of the KS keratinocyte cell lines (Figure 3.4).

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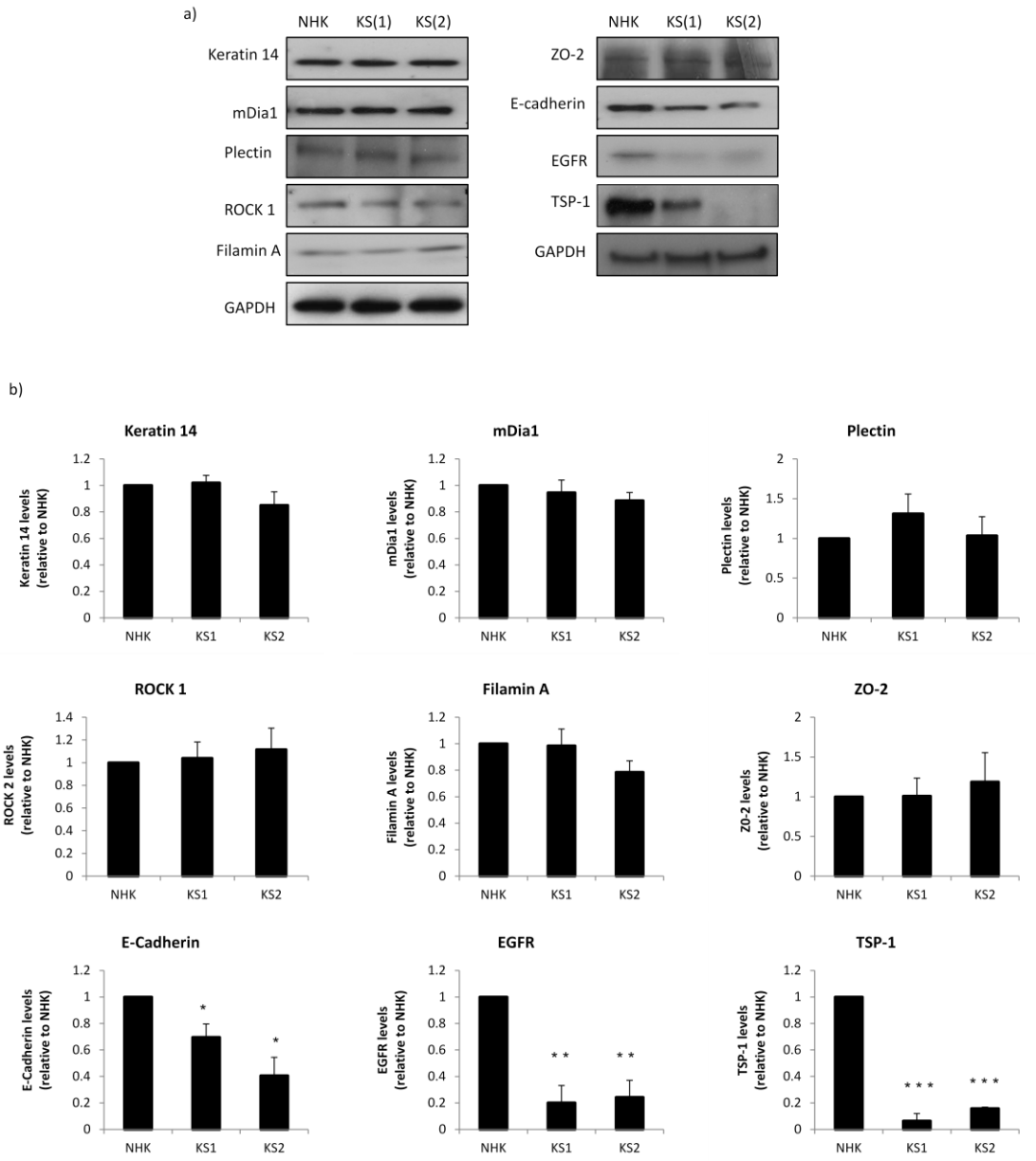


Figure 3.4: KS keratinocytes have lower levels of EGFR, TSP-1 and E-Cadherin.

Validation of mass spectrometry results using western blotting to compare total protein levels in NHK and two KS cell lines. a) example blots of western blots of the proteins keratin 14, mDia1, plectin, ROCK 1, filamin A, ZO-2, E-cadherin, EGFR and TSP-1 with GAPDH (loading control). b) Graphs show densitometry analysis of the corresponding proteins in NHK vs. KS keratinocytes. Error bars are S.E.M., n=3, * = P<0.05, * * = P<0.001, * * * = P<0.0001.

3.2.3 Kindlin-1 regulates the expression of TSP-1 in human keratinocytes

To validate and further investigate the downregulation of TSP-1, levels of TSP-1 mRNA and total protein were analysed using semi-quantitative RT-PCR and western blotting respectively (Figure 3.5 (a,b)). Results demonstrated no change in mRNA levels of TSP-1, but a significant reduction in the total TSP-1 protein levels in KS cells. To analyse the localisation of TSP-1 in skin, immunohistochemistry was performed on normal human skin and KS skin by staining fixed 5 μm frozen sections with an antibody against TSP-1 and DAPI. Confocal images demonstrated TSP-1 was highly localised to the dermal-epidermal junction (shown by white arrows) (Figure 3.5 (d)) in normal human skin. In contrast, TSP-1 staining appeared to be more disrupted in skin from KS patients compared to the normal skin although localisation was still most prominent at the dermal-epidermal junctions (Figure 3.5 (d)). To determine whether reduced TSP-1 levels seen in KS cells was due to increased TSP-1 in the insoluble fraction, cells were fractionated after lysing and split into soluble fraction (the supernatant) and the insoluble fraction (pellet). The fractions were analysed with western blotting and probed for TSP-1(Figure 3.5 (e)). As seen in previous soluble lysate analysis, TSP-1 levels were very low in the soluble fractions of KS keratinocytes but there was no corresponding increase in levels in the insoluble fractions.

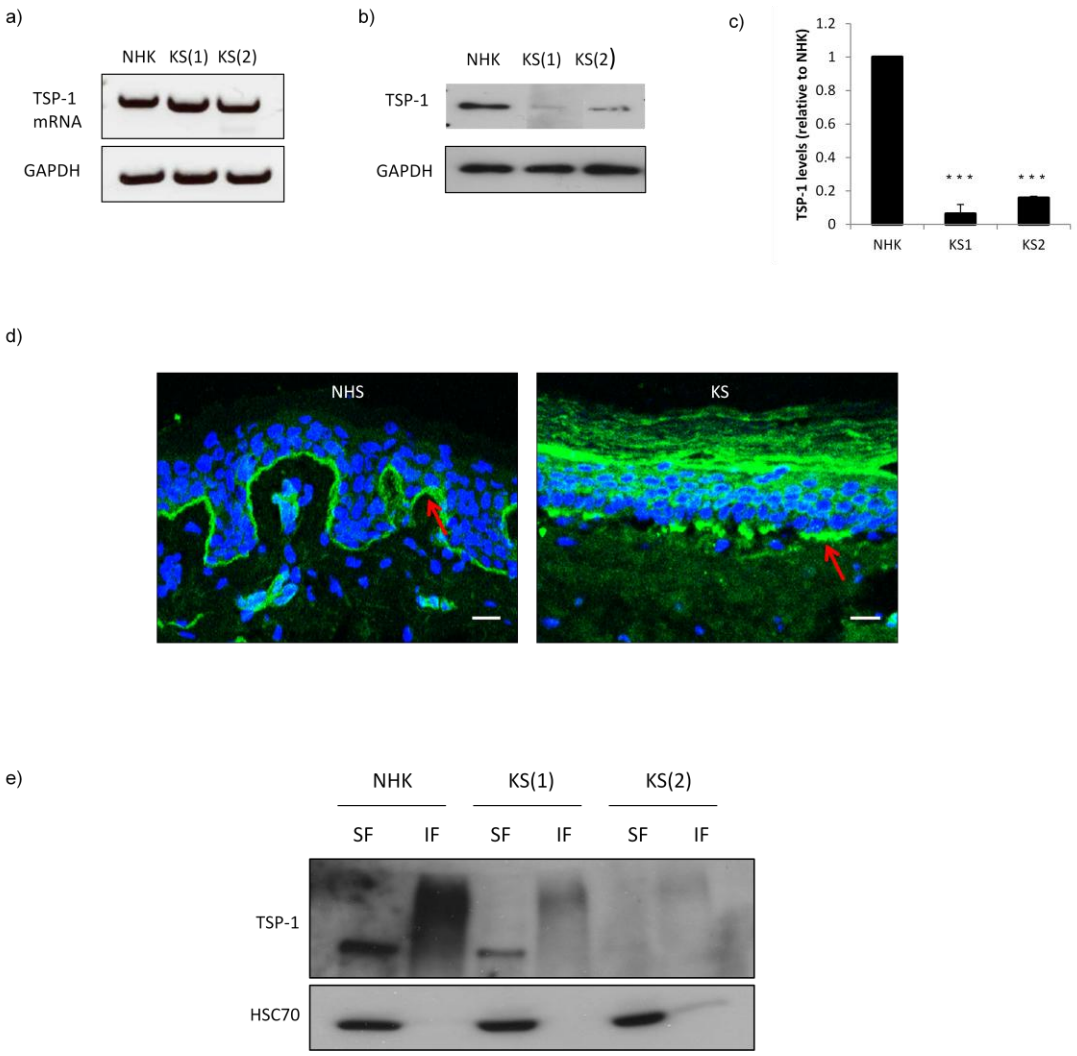


Figure 3.5: Characterisation of TSP-1 in keratinocytes and skin.

Characterisation of the gene and protein level and localisation of TSP-1 in NHK vs KS. a) Semi-quantitative RT PCR of TSP-1 mRNA levels in NHK and KS keratinocytes. b) Western blot example of TSP-1 total level in NHK vs. KS keratinocytes. c) Graphs show densitometry analysis of the protein. Error bars are S.E.M., n=3, * * * = P<0.0001. d) Confocal images of the localisation of TSP-1 (green) and DAPI (blue) in normal human skin (NHS) and KS patient skin. Arrows indicate localisation of TSP-1. Scale bar = 10 μm. e) Western blot of TSP-1 levels in soluble (SF) or insoluble fractions (IF) in NHK and KS keratinocytes.

3.2.4 TSP-1 is incorrectly deposited by KS cells

The localisation of TSP-1 in NHK and KS keratinocytes was determined by immunofluorescence microscopy. The cells were fixed and stained with antibodies against TSP-1 as well as α6-integrin, the latter being used as a marker of hemidesmosome adhesion complexes (Figure 3.6). TSP-1 was localised to the cell membrane and co-localised with α6-integrin staining in NHK. The protein was also secreted from NHK, which was seen as a layer of TSP-1 deposited around the cells in a rosette-like structure (Figure 3.6). Cells that were fixed in mid-migration also appeared to leave a trail of TSP-1 behind in the migration track. By contrast, KS keratinocytes showed much lower levels of TSP-1 deposited in the immediate surrounding area and no trail of TSP-1 was evident. However, TSP-1 could still be detected at the membrane of the KS cells. The difference in TSP-1 secretion was not quantified, however approximately 80% of KS keratinocytes analysed showed abnormal secretion and deposition of TSP-1. To investigate whether TSP-1 re-expression could rescue deposition and localisation in KS cells, KS keratinocytes were transfected with a cDNA construct encoding human TSP-1, fixed and stained with anti-TSP-1 antibodies (Figure 3.7). Transfected cells showed overexpression of TSP-1, but this TSP-1 still did not localise in a similar pattern to that seen in NHK keratinocytes and as seen befor around 80% percent of the cells were not secreting or depositing TSP-1 in a normal manner (Figure 3.7).

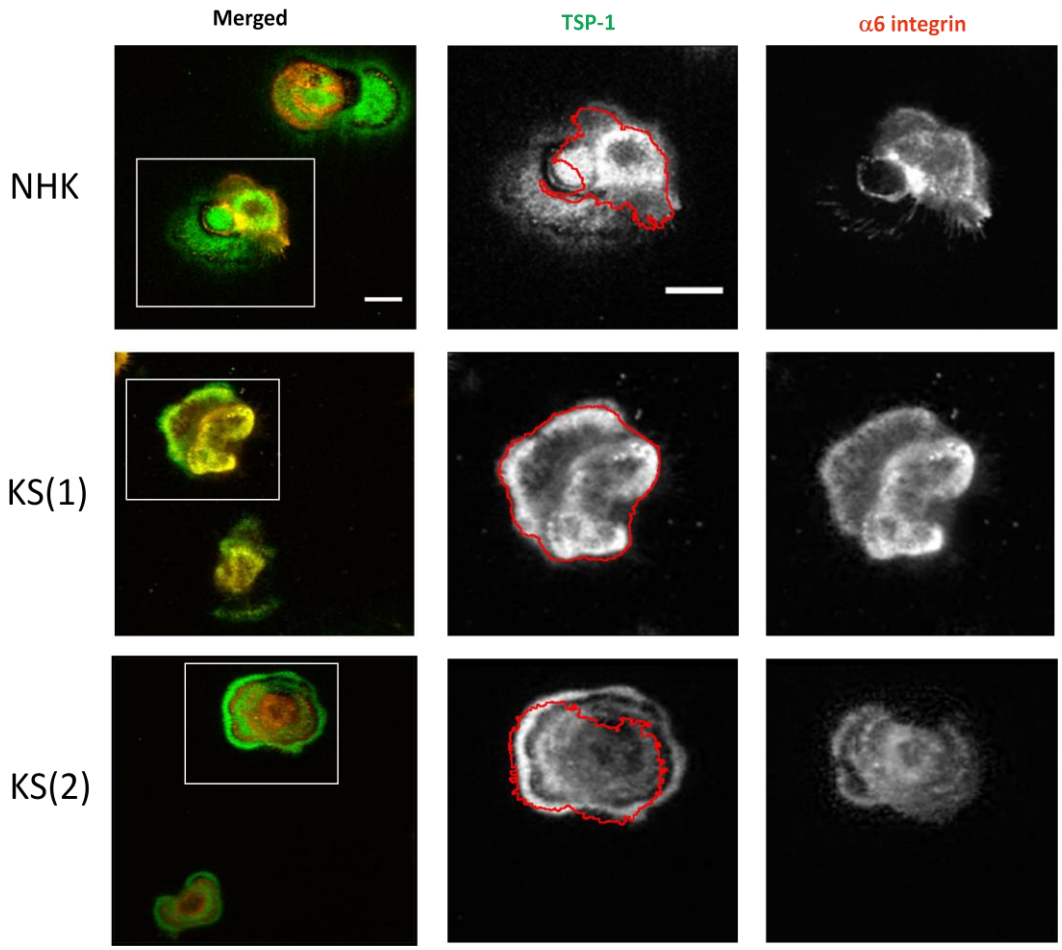


Figure 3.6: TSP-1 is retained at the membrane in KS keratinocytes.

Confocal images of fixed NHK or KS keratinocytes. TSP-1 is shown in green and α6 integrin in red. TSP-1 and α6 integrin channels have been zoomed in from the original image as highlighted by the white boxes. The red outline highlights the periphery of the cells. TSP-1 is secreted and deposited around the cells and the staining gives a rosette-like appearance. In KS keratinocytes there appears to be less secreted TSP-1 around the cells, with approximately 80% of cells analysed consisting of this phenotype. Scale bar = 10 μm.

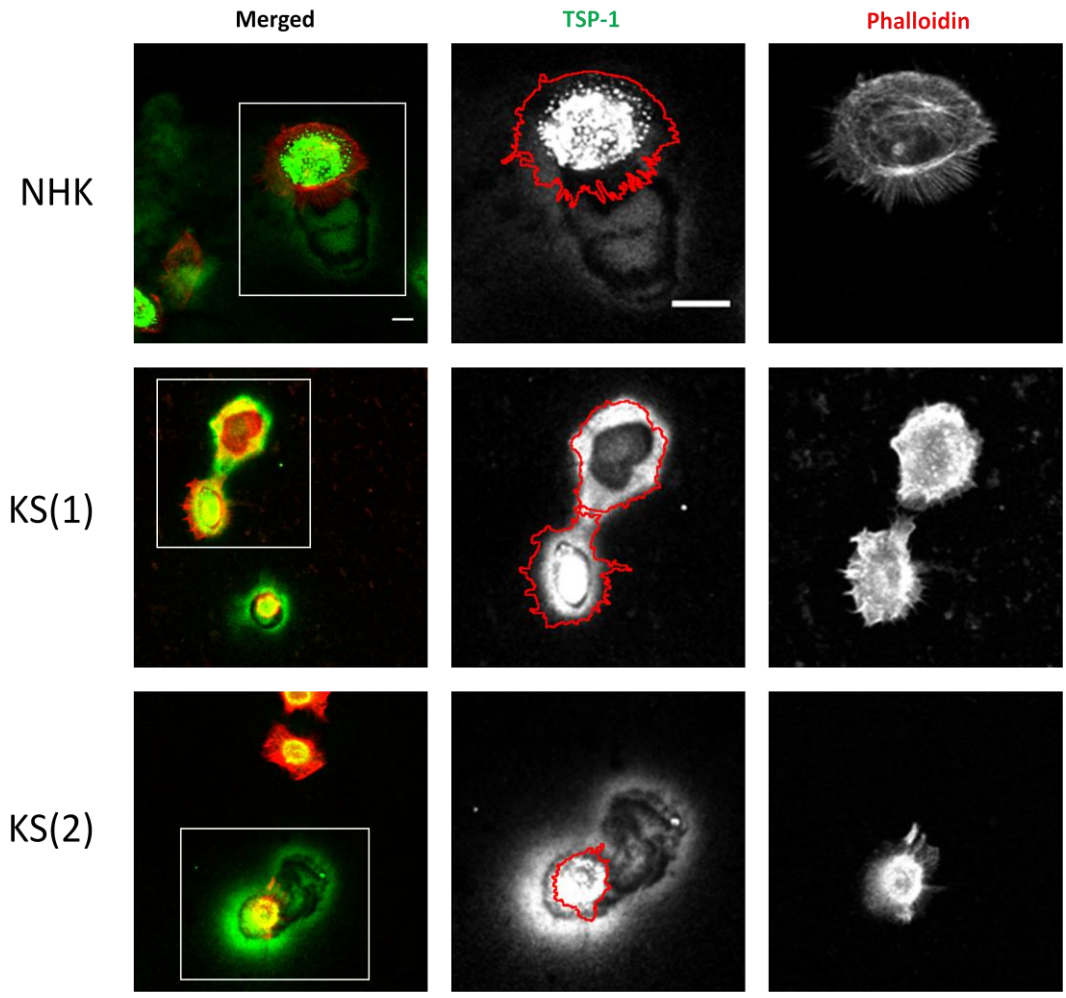


Figure 3.7: TSP-1 overexpression in KS keratinocytes does not restore normal TSP-1 deposition patterns. Confocal images of fixed NHK and KS keratinocytes overexpressing TSP-1. TSP-1 is shown in green and F-actin in red (Phalloidin 633). TSP-1 and phalloidin channels have been zoomed in from the original image as highlighted by the white boxes. The red outline highlights the periphery of the cells. The TSP-1 expressing KS keratinocytes were unable to secrete and deposit the protein similar to that seen in NHK cells. Scale bar = 10 µm.

3.2.5 Re-expression of kindlin-1 in KS keratinocytes rescues expression levels of TSP-1.

To confirm the role of kindin-1 in regulation of TSP-1 regulation, KS cell lines stably re-expressing kindlin-1 were generated. Wild type (WT) kindlin-1 and the non-integrin binding mutant Kindlin-1-W612A with an N-terminal mCherry tag (Figure 3.8 (a)) were cloned into lentiviral vector backbone to allow stable infection and re-expression in KS keratinocytes. FACS was used to sort the cells to achieve a pure population of mCherry positive cells. The level of TSP-1 in the KS keratinocytes expressing WT kindlin-1 or kindlin-1-W612A was determined using western blot analysis followed by densitometry analysis as shown in Figure 3.8 (b, c). KS keratinocytes expressing WT kindlin-1 showed a partial rescue of TSP-1 levels, although this was not significant. However, in the KS keratinocytes expressing kindlin-1-W612A there was a complete absence of TSP-1. The kindlin-1-mCherry expressing cells were further analysed using immunofluorescence after fixing and staining with TSP-1. The KS keratinocytes expressing WT kindlin-1 appeared to show rescue of TSP-1 deposition as more TSP-1 was secreted from the cells and was left as a track around the cells as they moved, this was seen in approximately 50-60% of the cells although quantification wss not performed (Figure 3.9). This rescue of TSP-1 localisation was not seen in the KS keratinocytes expressing kindlin-1-W612A as they did not exhibit normal TSP-1 deposition (Figure 3.9).

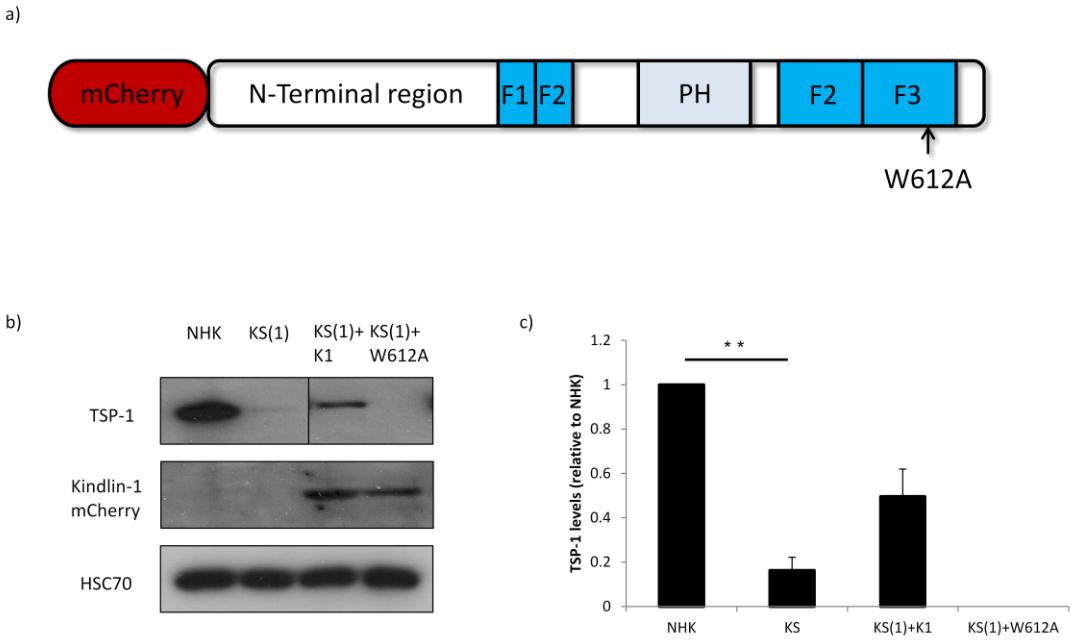


Figure 3.8: Kindlin-1 WT but not W612A mutant expression can partially rescue TSP-1 levels.

Genration of kindlin-1 rescue cell lines. a) Schematic of mCherry tagged kindlin-1 construct. Arrow indicates the location of the mutation W612A. b)Western blot analysis of TSP-1 total protein levels in NHK or KS keratinocytes stably expressing mCherry alone, mCherry WT kindlin-1 or mCherry tagged W612A kindlin-1 mutant. c) Graph shows densitometry analysis of TSP-1 protein levels. Error bars are S.E.M., n=3, * * = $P < 0.001$.

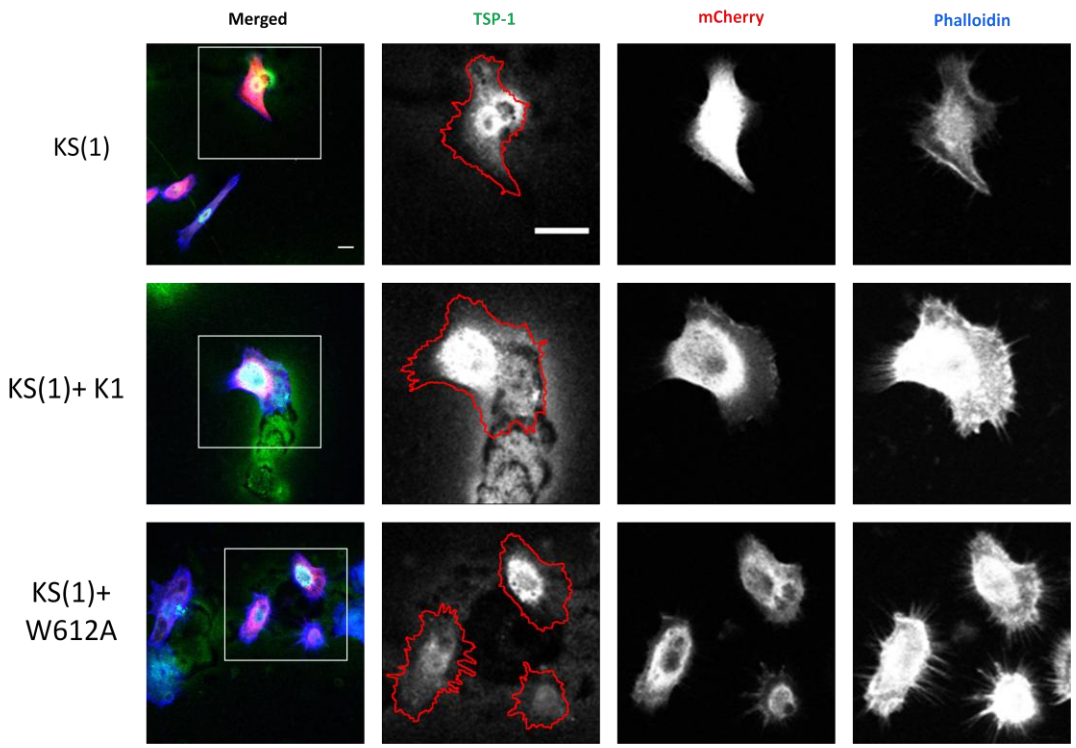


Figure 3.9: Deposition of TSP-1 is rescued by WT kindlin-1 but not by W612A mutant of kindlin-1.

Confocal images of fixed KS keratinocytes expressing mCherry, mcherry-WT kindlin-1 or mCherry kindlin-1-W612A. TSP-1 is shown in green, mCherry alone, WT kindlin-1 or kindlin-1-W612A in red and F-actin in blue (Phalloidin 633). TSP-1, mCherry and phalloidin channels have been zoomed in from the original image as highlighted by the white boxes. The red outline highlights the periphery of the cells. Approximately 50-60% of WT kindlin-1 expressing cells showed a rescue of the phenotype comparable to NHK, while kindlin-W612A expressing cells did not show any rescue of the phenotype. Scale bar = 10 µm.

3.3 Discussion

In this chapter I characterised the cell lines to be used in the project. Two KS patient cell lines with known mutations in kindlin-1 were chosen. KS(1) and KS(2) keratinocytes harbouring the following mutations, compound heterozygous p.E304X/p.L302X and homozygous c.676insC respectively. These mutations have been identified and characterised in previous studies (Ashton *et al.*, 2004). The use of two patient cell lines allowed comprehensive analysis of changes in protein expression relative to NHK cell line which was used as the control. The aberrant morphology of KS keratinocytes have previously been characterised by other groups, which have described the cells as having proliferation, adhesion, and migration defects (Herz *et al.*, 2006; Has *et al.*, 2009). The ability to bind and activate β1 integrin (Harburger *et al.*, 2009) allows kindlin-1 to participate in important integrin-mediated processes such as cell adhesion to underlying matrix, migration, cell polarisation. Hence kindlin-1 is important in controlling polarity, adhesion and maintenance of the cytoskeletal architecture in normal keratinocytes, and loss of kindlin-1 results in a range of associated defects.

Analysis of NHK and KS keratinocyte lysate using mass spectrometry identified a number of differentially-expressed proteins. The following proteins showed reduced expression in KS keratinocyts: Keratin 14, mDia1, plectin, ROCK2, filamin A, ZO-2, E-cadherin, EGFR and TSP-1. However, immunoblotting showed no comparative differences in these proteins with the exception of E-Cadherin, EGFR and TSP-1 indicating that the other findings were false positives, possibly due to the target bands being not well-resolved enough on SDS-PAGE gels and thus containing multiple proteins. Interestingly, the proteins that were validated to change expression

level are all associated with control of cell-cell or cell-matrix adhesion in skin biology.

E-Cadherin is an important cell-cell adhesion protein that has been implicated in cancer progression and metastasis due to loss of function or reduced expression (Gumbiner, 2005). Changes in E-Cadherin could possibly be related to the increased susceptibility of squamous cell carcinoma that is a clinical feature in KS patients. Due to time constraints I did not further investigate the link between kindlin-1 and E-Cadherin. However, this will be an important topic to analyse in future studies.

TSP-1 was another protein found to be significantly reduced in KS keratinocytes. TSP-1 is a matricellular protein that mediates cell-cell and cell-matrix interactions. It is also a potent inhibitor of angiogenesis and is also found to be reduced in photoageing skin (Yano *et al.*, 2005; Kim *et al.*, 2006). This may explain some of the telangiectasiae and atrophy that is evident in the skin of many KS patients. Immunofluorescence of NHK and KS keratinocytes with TSP-1 showed that TSP-1 was secreted and deposited around the cells, whereas in KS cells (KS(1) and KS(2)) TSP-1 is retained at the cell membrane. This suggests that there is a disruption in the secretory mechanism of the KS cells. Overexpressing TSP-1 in KS cells was unable to rescue normal deposition implying a defect in the regulation of TSP-1. Expression of wild type kindlin-1 was able to partially rescue expression levels in KS keratinocytes, as well as rescuing normal deposition of TSP-1. However, this was not seen when the W612A non-β1 binding mutant of kindlin-1 was re-expressed in KS cells. In fact, TSP-1 was completely absent from these cells and expression seemed to be repressed. The ability of wild type but not the W612A kindlin-1 to rescue TSP-1 levels and secretion confirms that kindlin-1 is involved in TSP-1 regulation and further suggests that this regulation depends on integrin binding to

kindlin-1. It is possible that TSP-1 deposition requires fully active β1 integrins, and kindlin-1 is required for this step. Alternatively (or additionally), kindlin-1:integrin binding may control a signalling pathway that promotes TSP-1 protein production and secretion. These would be interesting hypotheses to test in future studies.

Taken together, these results have identified a number of novel targets that are differentially expressed in KS keratinocytes. Results also show that kindlin-1 is important for the expression and secretion of TSP-1. The role of kindlin-1 in regulating EGFR expression will be further explored in Chapter 4.

Chapter 4:

Kindlin-1 regulates EGFR expression and signalling

4.1 Introduction

Integrin receptors independently propagate signals in cells, however they are also able to coordinate with other transmembrane receptors such as receptor tyrosine kinases (RTK). The crosstalk between these two receptor types is vital for normal development, tissue homeostasis, cell proliferation, survival and migration (Alexi *et al.*, 2011). The co-operation can occur at the level of integrin-dependent activation of RTK, recruitment of transducing proteins to membrane cytoskeletal complexes and increased nuclear localisation of transcriptional regulators (Eliceiri, 2001). RTK activate a multitude of signalling pathways including PI3K and MAPK which are important for cell proliferation and survival (Bazley and Gullick, 2005). There are different mechanisms of crosstalk between integrins and RTK. Growth factors that bind and activate RTK can alter integrin localisation and activation thereby regulating integrin mediated events such as cell adhesion, spreading and migration (Cabodi *et al.*, 2004). Conversely, integrin signals are needed for enhanced activation of RTK. In epithelial cells, β1 integrin has been shown to activate EGFR and is also required for full activation in response to EGF leading to transcriptional changes (Cabodi *et al.*, 2004). Adhesion of epithelial cells to ECM is sufficient to stimulate EGFR and activate the Akt and Erk pathways which lead to upregulation of cyclin D1 for cell cycle progression (Bill *et al.*, 2004). EGFR belongs to the family of ErbB RTK, which consists of four members. EGFR (ErbB1) exists on the cell surface and is activated by binding of its specific ligands, including epidermal growth factor (EGF), transforming growth factor α (TGFα) and heparin binding EGF (HB-EGF) (Bazley and Gullick, 2005). EGFR is the main member of the ErbB family that is expressed in keratinocytes in skin. Upon activation by its growth factor ligands, EGFR undergoes transition from an inactive monomeric form to an active

homodimer. In addition to forming homodimers after ligand binding, EGFR may also form heterodimers with another member of the ErbB receptor family (Bazley and Gullick, 2005). There is also evidence to suggest that clusters of activated EGFRs form, although it remains unclear whether this clustering is important for activation itself or occurs subsequent to activation of individual dimers. EGFR dimerisation stimulates its intrinsic intracellular protein-tyrosine kinase activity and this causes autophosphorylation of several tyrosine residues in the C-terminal domain of EGFR. Autophosphorylation elicits downstream signalling that involve other proteins that associate with the phosphorylated tyrosines through their own phosphotyrosine-binding SH2 domains. These downstream signalling proteins initiate several signal transduction cascades, principally the MAPK, Akt and JNK pathways and these pathways modulate phenotypes such as cell migration, adhesion, and proliferation (Bazley and Gullick, 2005). Inactivation of EGFR is achieved through dephosphorylation by phosphotyrosine phosphatases or receptor downregulation. Downregulation of EGFR occurs when ligands bind to the receptor leading to its internalisation and subsequent degradation. Unbound EGFR is recycled back to the cell surface, while the ligand bound receptors are degraded in the lysosome by proteases (Authier *et al.*, 1999). EGFR has also been shown to be degraded through another pathway. Ubiquitination of EGFR is mediated by the c-Cbl protein, which possesses a SH3 domain that interacts with phosphorylated tyrosine on the EGFR cytoplasmic domain (Joazeiro *et al.*, 1999). This propagates a signal for degradation by the 26S proteasome (Ettenberg *et al.*, 2001). Reports suggest that integrins may have a role in regulating EGFR recycling, for example chemically inhibiting β3 integrin increases the recycling of EGFR and α5β1 where both can form a complex and be endocytosed together (Caswell *et al.*, 2008). The

aim of this chapter was to further characterise the kindlin-1-dependent regulation of EGFR levels identified in the previous results chapter, and to determine how kindlin-1 may act co-operatively with EGFR to maintain normal keratinocyte signalling responses to EGF.

4.2 Results

4.2.1 EGFR is downregulated in KS keratiniocytes

Mass spectrometry analysis initially showed a reduction in protein levels of EGFR in KS keratinocytes. However, semi-quantitative RT-PCR detected no change in the mRNA level of EGFR as demonstrated in Figure 4.1 (a). To confirm changes in total protein level immunoblotting was performed (Figure 4.1 (b), which showed a significant reduction in levels of EGFR in both KS cell lines. Furthermore, FACS analysis revealed a significant reduction in cell surface levels of EGFR also in both KS cell lines (Figure 4.1 (c)). Localisation of EGFR was analysed by imuunostaining normal human skin (NHS) and KS skin with an anti-EGFR antibody, followed by confocal analysis. KS skin showed an almost complete absence of EGFR in the basal keratinocytes, but not in NHS.

To analyse the localisation of EGFR in KS keratinocytes, the cells were fixed and stained with an anti-EGFR antibody alongside F-actin staining. Confocal images were taken of NHK and KS keratinocytes for comparison (Figure 4.2). In NHK, EGFR localised partially at the membrane and also at some perinuclear vesicular structures as has been reported previously in other cells types. By contrast, EGFR staining in KS keratinocytes was very low at the membrane and increased within perinuclear vesicles (Figure 4.2).

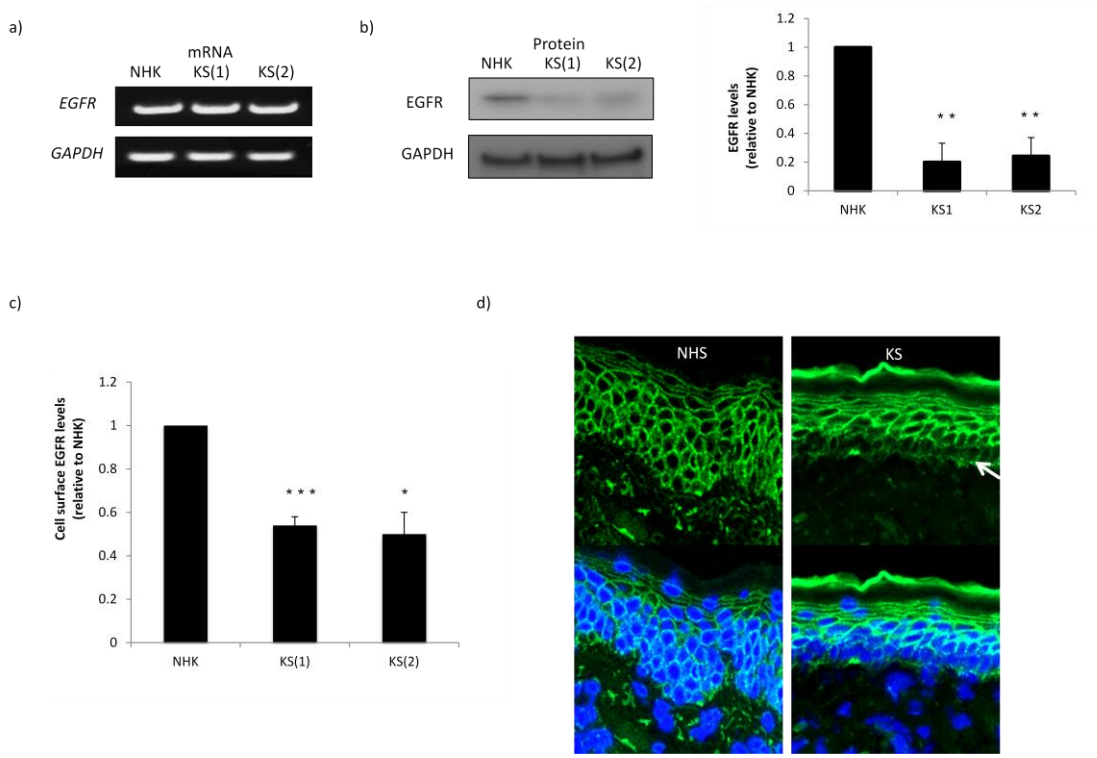


Figure 4.1: EGFR levels are reduced in KS cells

Characterisation of protein and mRNA levels of EGFR in NHK vs. KS keratinocytes a) Semi-quantitative RT-PCR of *EGFR* mRNA levels in NHK and KS keratinocytes. b) Western blot example of EGFR total level in NHK vs. KS keratinocytes. Graph shows densitometry analysis of the protein Error bars are S.E.M., n=3, * * = P<0.001. C) Quantification of surface levels of EGFR in NHK and KS keratinocytes by FACS analysis. Error bars are S.E.M., n=3, * = P<0.05, * * * = P<0.0001. d) Confocal images of EGFR (green) and DAPI (blue) in normal human skin (NHS) and KS skin. Arrow indicates the basal keratinocytes in KS skin.

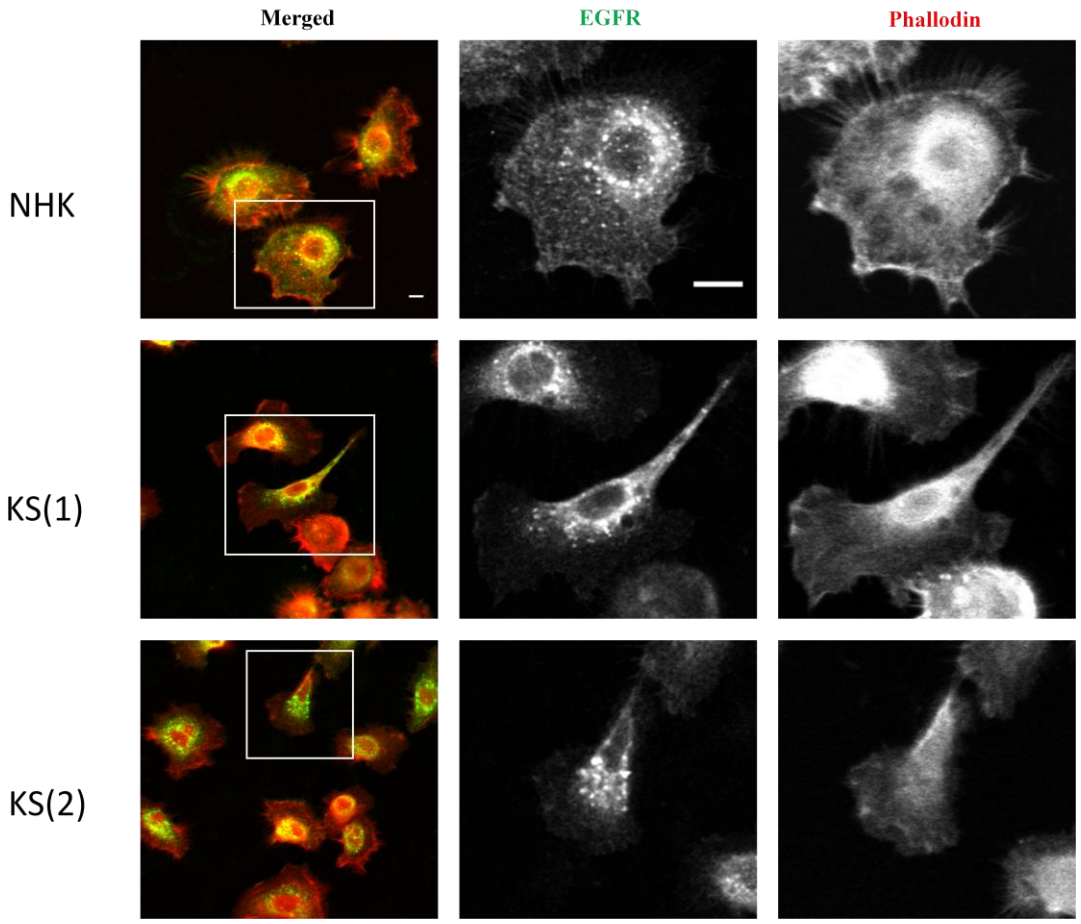


Figure 4.2: In KS keratinocytes EGFR localises more to the nuclear periphery than at the membrane.

Confocal images of fixed NHK and KS keratinocytes. Endogenous EGFR is shown in green and F-actin in red (Phallodin 633). EGFR and phalloidin channels have been zoomed in from the original image as highlighted by the white boxes. Scale bar = 10 μm.

4.2.2 KS keratinocytes show altered response to EGF

To examine the effects of EGF stimulation on EGFR localisation, cells were starved overnight in OPTI-MEM (that contains no growth factors) and then stimulated with EGF (100 ng/ml). Cells were fixed at 0 min, 15 min or 60 min after EGF treatment and stained with anti-EGFR antibody followed by analysis by confocal microscopy (Figure 4.3). At 0 min EGF treatment (i.e. starved cells), NHK cells showed EGFR localisation at both the plasma membrane and cytoplasm, and after 15 min of EGF stimulation there was lower EGFR at the membrane and more in discrete punctate vesicles within the cytoplasm, some of which were immediately beneath the plasma membrane. After 60 min, EGFR was almost completely absent from the plasma membrane but showed more punctate localisation in the cytoplasm in vesicles, mainly around the perinuclear region. Moreover, staining for F-actin demonstrated a clear increase in peripheral protrusions in NHK after EGF treatment, and polarisation of the cell at 60 minutes. In KS keratinocytes there were no obvious changes in EGFR localisation in response to EGF stimulation. In starved cells there was high perinuclear staining of EGFR that did not change following 15 minutes or 60 minutes of EGF stimulation.

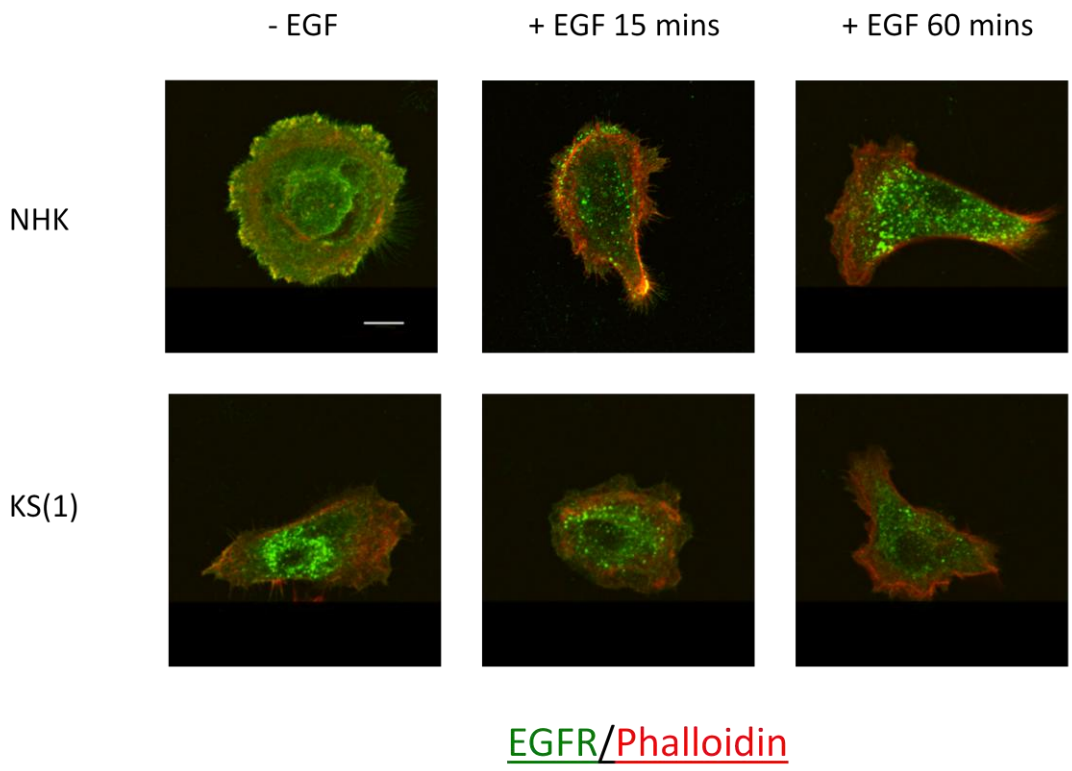


Figure 4.3: EGFR is internalised after EGF treatment in NHK.

Confocal images of NHK or KS(1) keratinocytes treated with EGF (100 ng/ml) for 15 or 60 mins and stained for EGFR, shown in green and F-actin in red (Phalloidin 633).Scale bar = 10 μm.

To further investigate the regulation of EGFR in response to EGF stimulation in NHK and KS cells, the total and plasma membrane associated levels of EGFR was measured by intensity analysis of confocal images before and after EGF stimulation. Example confocal images of the cells used to quantify total and surface EGFR before and after stimulation are shown in Figure 4.4 (a). Laser levels and detector settings were maintained at identical levels for all images acquired to permit direct comparison. The total level of EGFR did not change in response to EGF stimulation in either NHK or KS keratinocytes as demonstrated by total EGFR intensity quantification in Figure 4.4 (b). However, as seen by western blotting, there was almost half the level of EGFR in KS vs. NHK keratinocytes. The surface level of EGFR significantly decreased in NHK after 60 minutes EGF stimulation. However, surface levels of EGFR in KS keratinocytes were reduced to a significantly lower level after EGF stimulation compared to that seen in NHK (Figure 4.4 (c)).

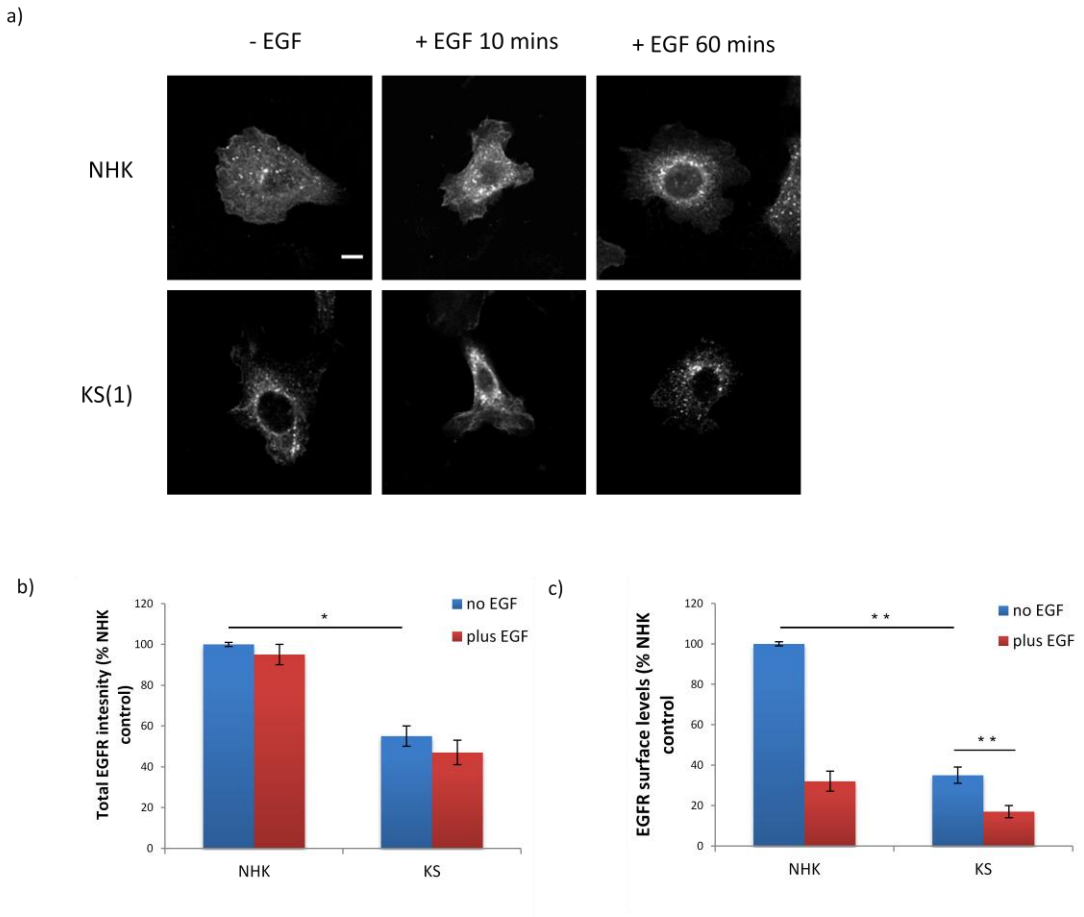


Figure 4.4: EGFR surface levels and intensity reduced in KS cells.

a) Example confocal images of EGFR staining in cells treated with EGF over time. Quantification of b) total and c) surface EGFR levels in cells at time 0 or 60 mins post-EGF by intensity analysis (graphs). n= >15 cells for each pooled from two independent experiments. Error bars are S.E.M., * = P< 0.01, * * = P< 0.001.

Given the striking changes in EGFR levels and response of KS cells to EGF, I next analysed whether EGFR signalling may be defective in KS cells. EGFR is known to undergo multiple tyrosine phosphorylation events at the cytoplasmic domain in response to EGF stimulation. To analyse this, EGFR was immunoprecipitated from NHK and KS lysates before and after EGF stimulation (10 min at 100 ng/ml) followed by western blotting and probing with anti-phophotyrosine antibody (Figure 4.5 (a)). Data showed that EGFR was being phosphorylated at tyrosine residues in response to EGF in both NHK and KS cells. However, KS cells showed a possible decrease in levels of phospho-EGFR corresponding to the lower level of EGFR that is immunoprecipitated in these cells.

To further analyse whether EGFR downstream signalling after EGF stimulation was also occurring in KS cells, immunoblotting was used to compare lysates from starved NHK and KS cells and those treated with EGF (10 min at 100 ng/ml). Two key proteins in the different signalling pathways of EGFR was analysed, including Akt and phospho-Akt or Erk1/2 and phospho-Erk1/2. This revealed no significant changes in total Akt, phospho-Akt or total Erk1/2. However, strikingly, KS cells showed no increase in phospho-Erk1/2 after stimulation with EGF whereas a robust response was seen in NHK. This was seen in both KS cell lines (Figure 4.5 (b)).

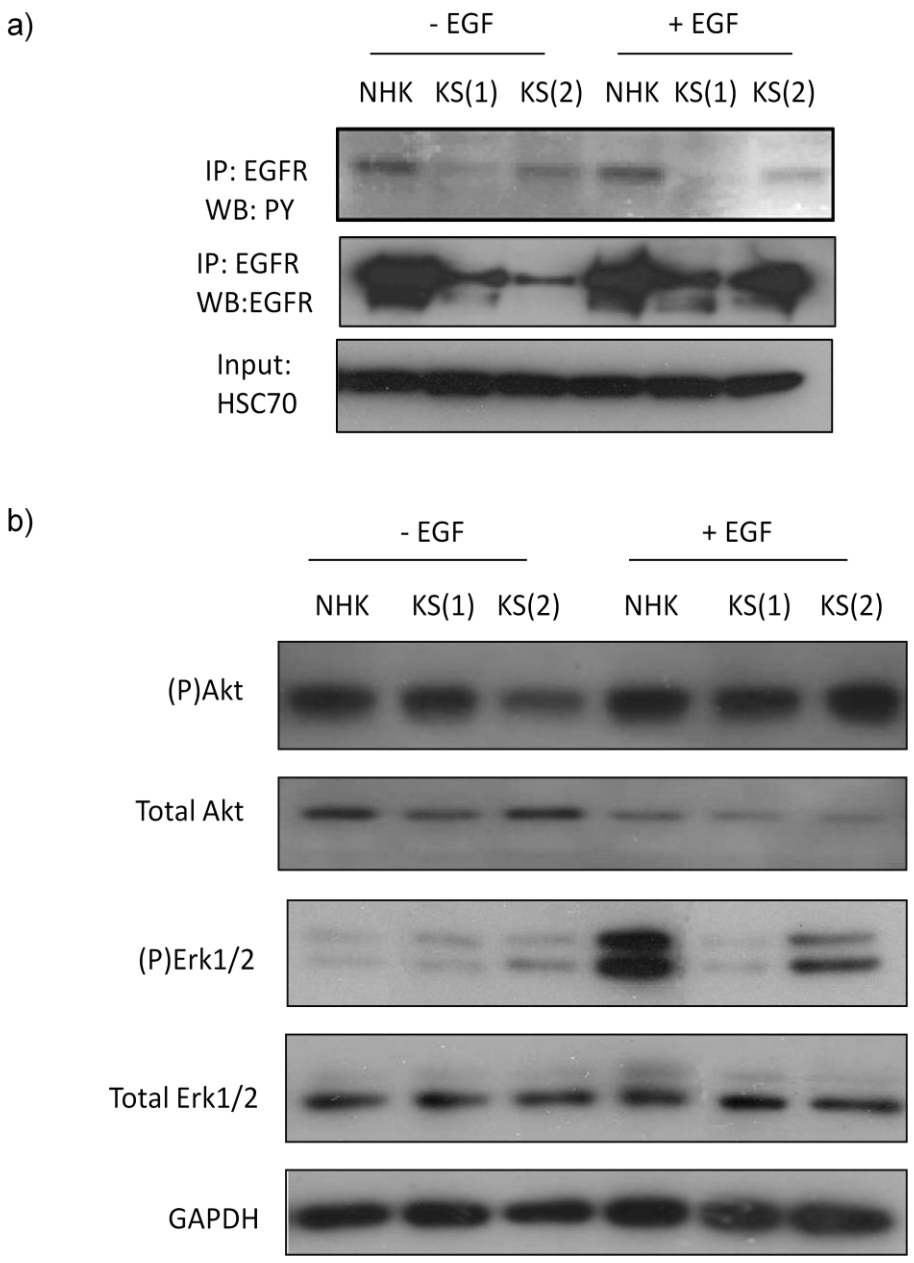


Figure 4.5: EGFR signalling is defective in KS keratinocytes.

a) Immunoprecipitation of EGFR from lysates from NHK and KS keratinocytes -/+EGF (100ng/ml, 10mins) and probed for phosphotyrosine (top panel; PY), EGFR or HSC70 (input only). b) Western blots of lysates from NHK and KS -/+ EGF (100 ng/ml, 10 mins) probed for phospho or total Akt and Erk1/2. GAPDH was used as a loading control.

To investigate the functional response of KS keratinocytes to EGF stimulation, phase contrast time lapse microscopy was employed to observe any changes in behaviour. NHK and KS keratinocytes were imaged lived before and after treatment with EGF as illustrated by the still images taken from time-lapse movies (Figure 4.6 (a)). As EGF is known to induce cell rounding followed by re-spreading in keratinocytes, the cell area was measured and quantified as a percentage relative to time 0 min in both cells lines (Figure 4.6 (b)). Both NHK and KS keratinocytes immediately contracted after EGF stimulation and at 4 min the percentage cell area was less than half that seen before stimulation. From this time point the cells began to re-spread again and by 10 mins post-EGF the NHK cells had reached 80% of initial cell area size. However, the KS keratinocytes demonstrated delayed re-spreading and only reached 50% of the original cell area size by 10 min.

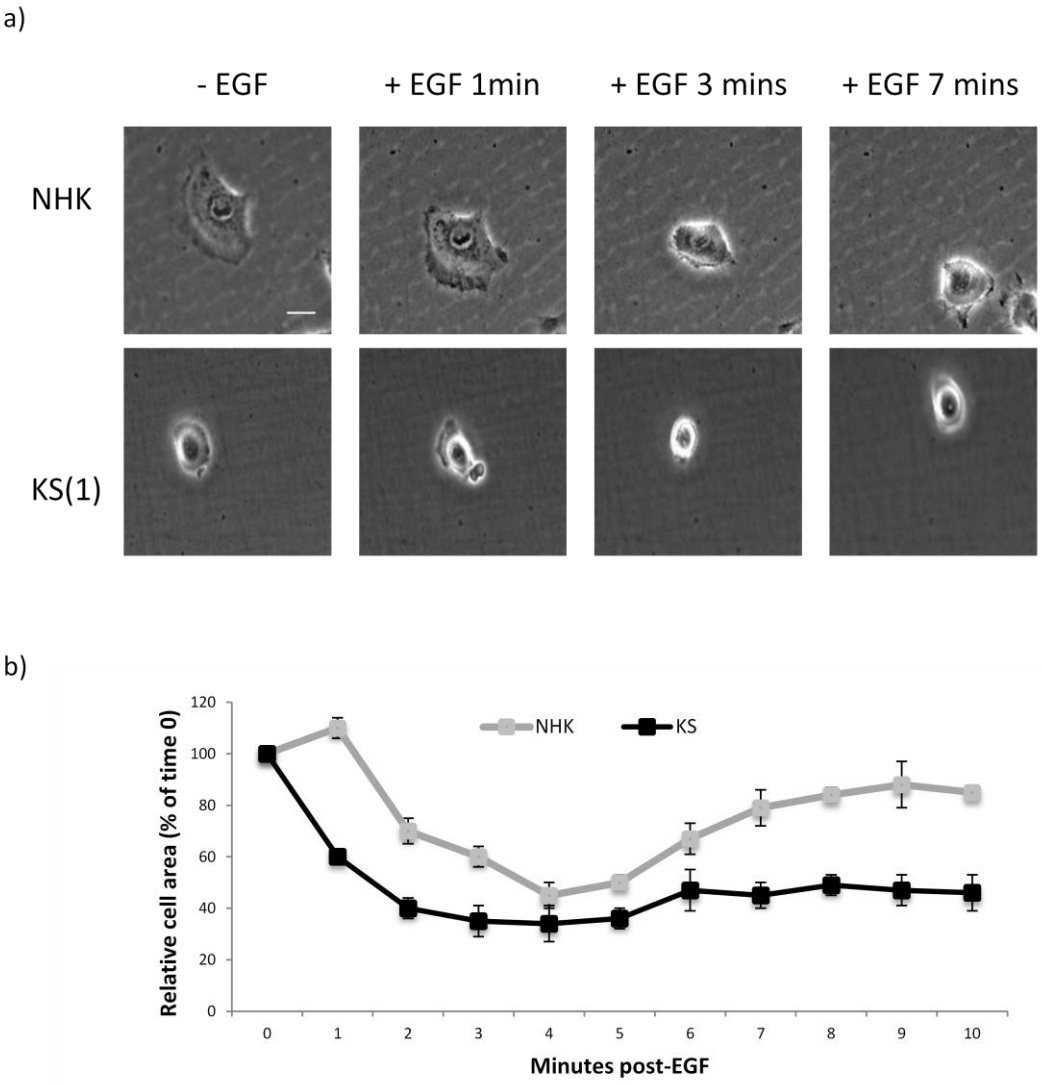


Figure 4.6: KS Keratinocytes show delayed re-spreading after EGF stimulation.

a) Still images of NHK or KS keratinocytes taken from time-lapse movies in cells pre and post EGF (100ng/ml). b) Graph shows quantification of spread cell area relative to pre-EGF treatment in >15 cells per condition over time. Error bars are S.E.M.

To further analyse the differential response of NHK and KS cells to EGF, time-lapse confocal microscopy was employed to study EGFR-GFP localisation in NHK and KS keratinocytes before and after EGF treatment over time. Still images from example movies are shown in Figure 4.7 from time points 0, 3, 7 and 11 mins post-EGF. In starved cells, GFP-EGFR appeared to localise predominantly at the plasma membrane and in some vesicles in NHK cells, as seen in staining for endogenous EGFR. KS cells, however, showed higher levels of EGFR-GFP localised within cytoplasmic vesicles. After stimulation with EGF, GFP-EGFR in NHK began to move into the cell as it was internalised from the plasma membrane, as seen by an increased level of punctate GFP-EGFR localisation in the centre of the cell. This internalisation was very rapid and was observed by 3 mins post-EGF and progressively increased over the 11 mins time range. By comparison GFP-EGFR localisation in KS keratinocytes did not significantly change following EGF treatment as the receptor was mainly localised within the cell in vesicles rather than the plasma membrane throughout the imaging time. However, by 7 mins there appeared to be a slight increase in levels of GFP-EGFR within perinuclear compartments suggesting that some EGFR was being internalised.

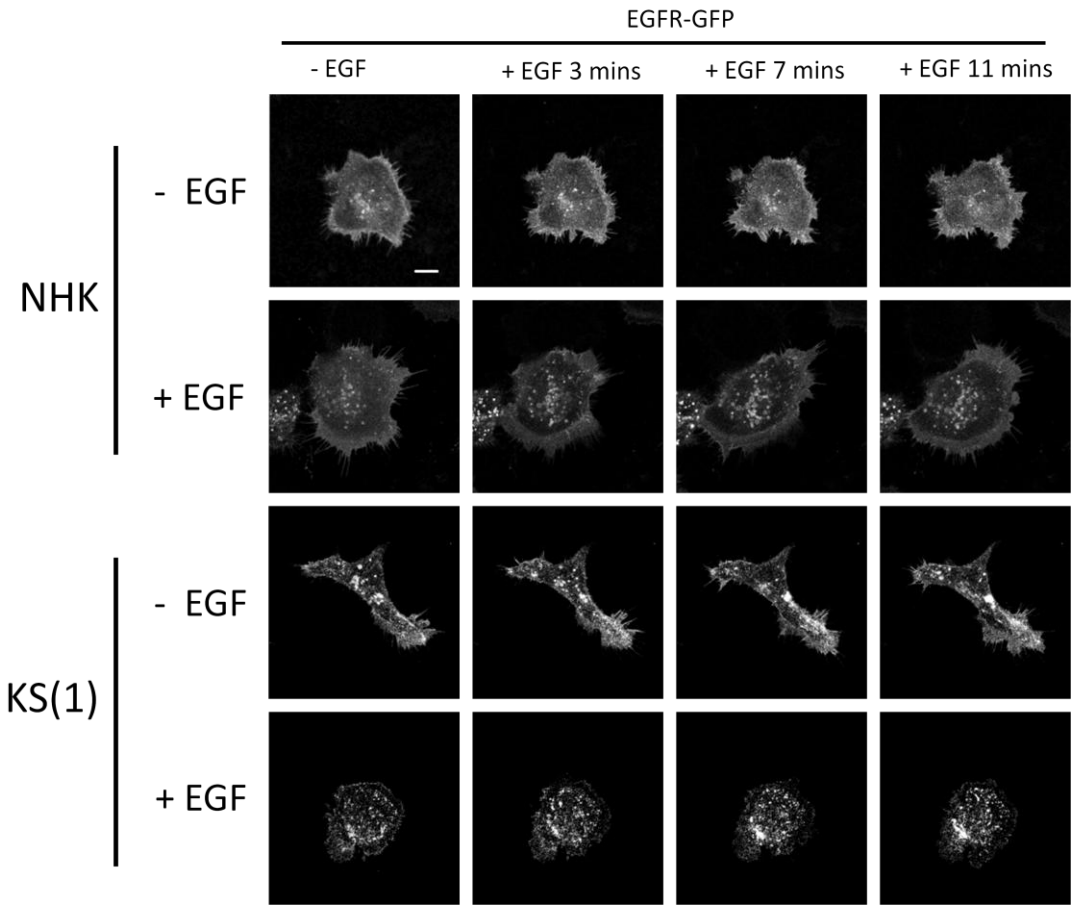


Figure 4.7: Live imaging of EGFR in KS keratinocytes shows slow response to EGF

Still images taken from time-lapse confocal movies of NHK or KS cells expressing EGFR-GFP with or without EGF treatment over time. Scale bar = 10 μm.

EGF is known to promote migration in human keratinocytes. To analyse whether the differences seen in the functional response of NHK and KS keratinocytes to EGF also lead to altered migration, cells were subjected to phase contrast time-lapse microscopy before and after treatment with EGF. Cells were tracked in movies and the migration speed and persistence was measured and plotted in the graphs shown in Figure 4.8 (a). Data demonstrated a significant increase in migration speed in KS cells compared to NHK in the absence of EGF. NHK responded to EGF stimulation by showing an increase in migration speed and persistence. However, there were no significant changes in speed or persistence by KS keratinocytes after EGF stimulation, suggesting a defect in the response and signalling mechanism in these cells (Figure 4.8 (b, c)).

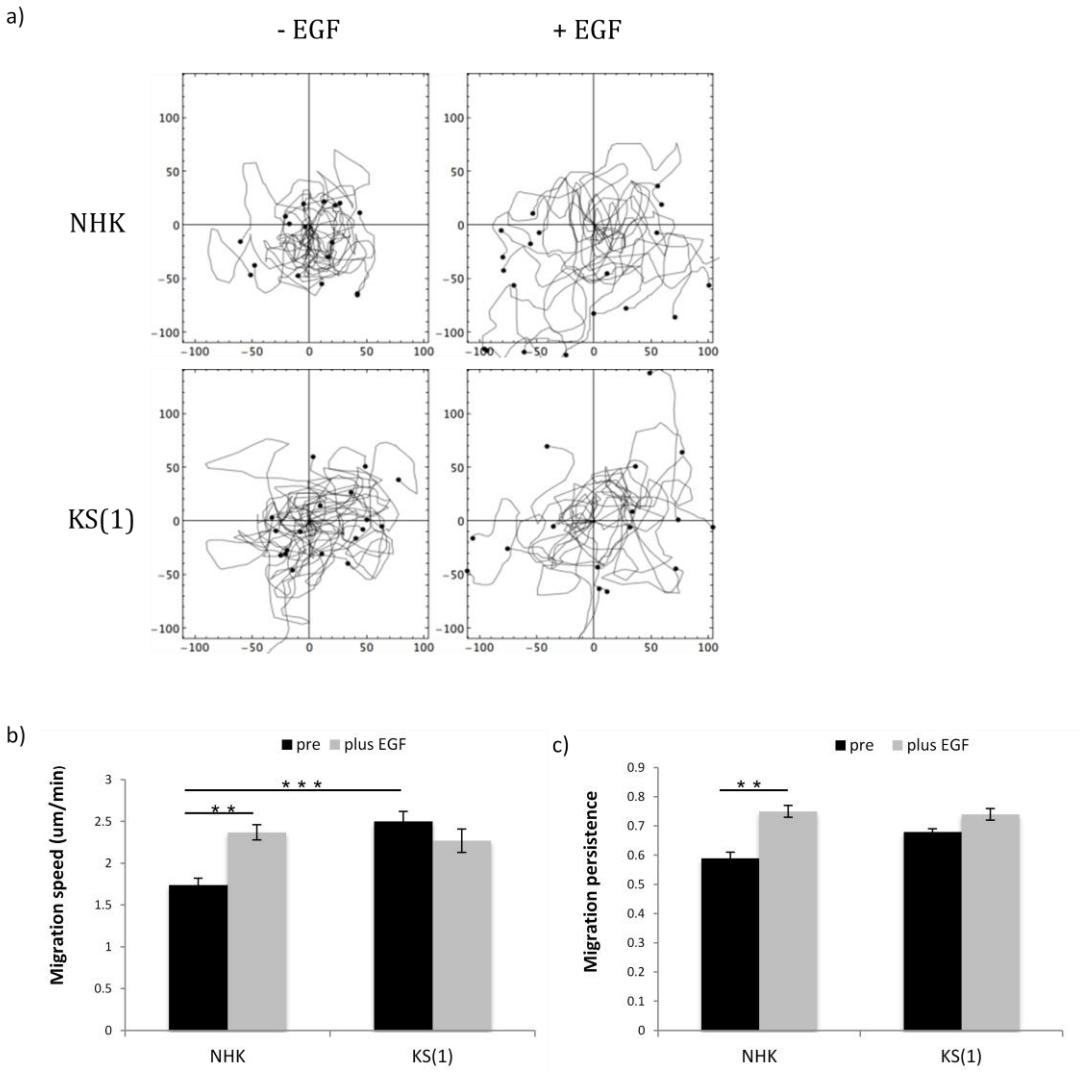


Figure 4.8: KS keratinocytes migration speed and persistence does not alter after EGF stimualation.

a) Representative tracks of NHK or KS keratinocytes undergoing migration before or after EGF stimulation (100ng/ml). Graphs of quantification from >60 cells of b) migration speed or c) persistence. Error bars are S.E.M., * * = P< 0.001, * * * = P< 0.0005.

4.2.3 Kindlin-1 regulates EGFR degradation

As the reduced protein level of EGFR could not be explained by downregulation at the gene level, it was suspected that EGFR was being degraded in the KS cells. It has been reported that the proteasome plays a role in the degradation of ubiquitinated EGFR and use of a proteasomal inhibitor, such as MG132, can prevent the degradation of EGFR and result in its accumulation (Ettenberg *et al*., 2002; Longva *et al*., 2002). Thus, NHK and KS keratinocytes were treated with the proteasomal inhibitor MG132 to determine whether this could rescue levels of EGFR in KS keratinocytes and confirm that EGFR was being rapidly degraded in these cells. Figure 4.9 (a) shows a representative western blot of EGFR levels in NHK and KS keratinocytes before and after treatment with MG132. From this data it is clear that EGFR is not being rescued from degradation in either of the KS cell lines using MG132. A further mechanism known to regulate EGFR degradation is the lysosomal pathway (Authier *et al.*, 1999). Degradation of a protein via the lysosome can be inhibited by the compound leupeptin. Treatment of KS keratinocytes with leupeptin was able to fully rescue levels of EGFR in one of the KS cell line and only partially in the other (Figure 4.9 (b)).

To determine whether EGFR was also seen in the lysosomal compartment in KS cells, cells were fixed and stained for the lysosomal marker LAMP-1 and EGFR and analysed by confocal microscopy. Images were subjected to Pearson's correlation colocalisation analysis to determine whether more EGFR resided in this compartment in KS cells. Analysis revealed that there was increased colocalisation of EGFR with the lysosomal marker LAMP-1. Pearsons's colocalisation co-efficient showed that colocalisation between EGFR and LAMP-1 was twice as high in KS compared to NHK (NHK 0.29+/-0.04 vs KS 0.64+/-0.08). Example confocal images

of the NHK and KS keratinocytes stained with EGFR and LAMP-1 can be seen in Figure 4.10.

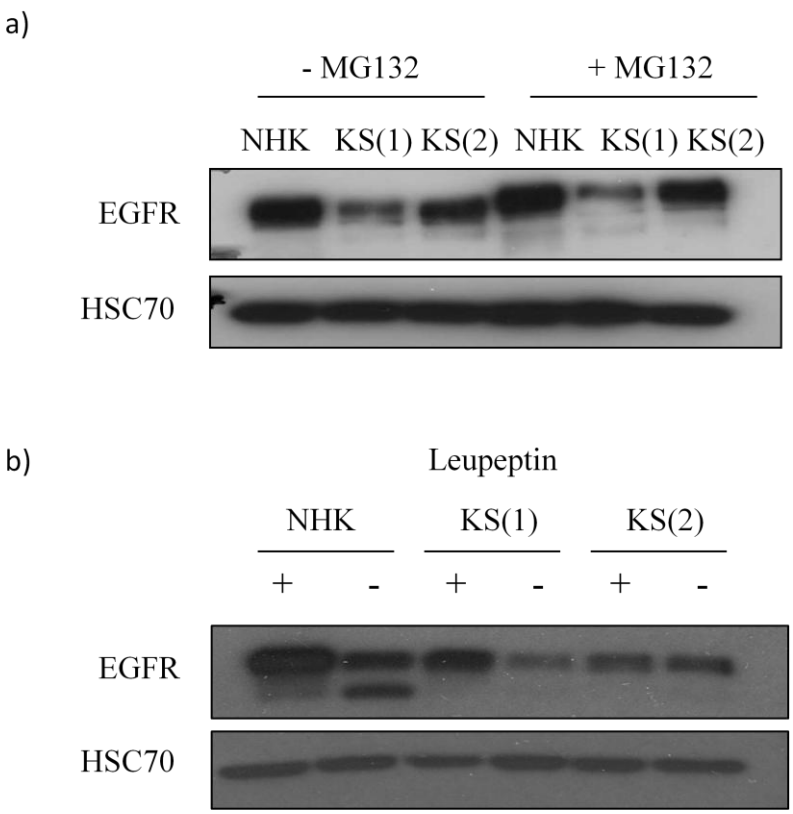


Figure 4.9: EGFR is being degraded via the lysosomal pathway in KS keratinocytes.

a) Western blot of EGFR total protein levels in NHK and KS keratinocytes before and after treatment with the proteasomal inhibitor MG132. HSC70 used as a loading control. b) Western blot of EGFR total protein levels in NHK and KS keratinocytes before and after treatment with the lysosomal inhibitor leupeptin. HSC70 used as a loading control.

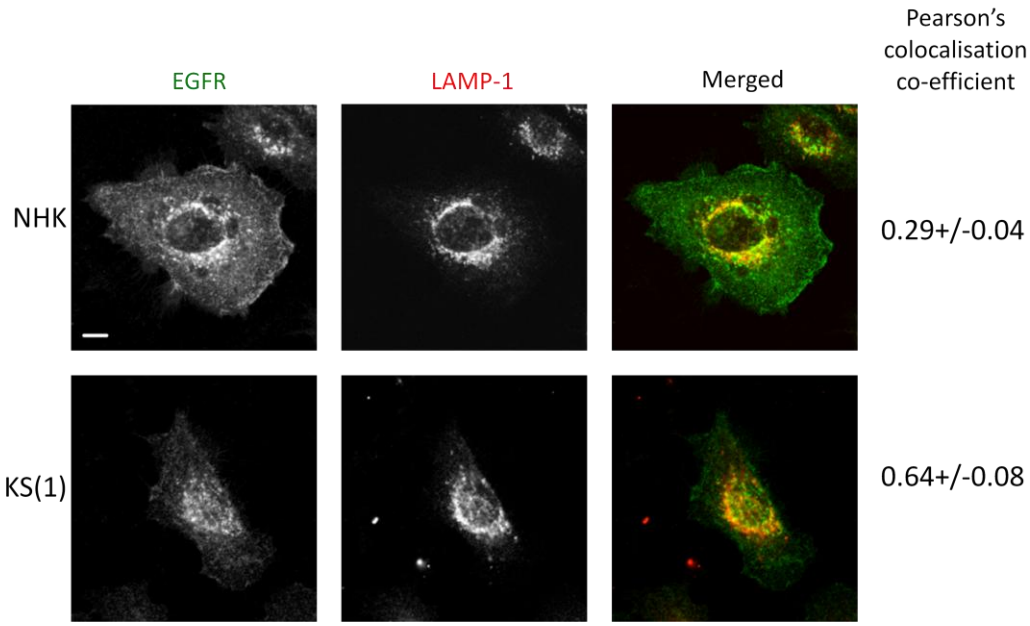


Figure 4.10: Increased colocalisation of EGFR with the lysosomal marker LAMP1 in KS keratinocytes.

Representative confocal images of NHK or KS keratinocytes stained for EGFR, shown in green and LAMP-1 in red. Scale bar = 10 μm. Images were analysed for colocalisation using Pearson's correlation co-efficient analysis (% colocalisation shown next to merged images +/-S.E.M).

4.2.4 Rescue of EGFR levels and response to EGF in KS cells

In order to confirm that the loss of EGFR in KS cells was directly due to kindlin-1 expression, WT kindlin-1 and kindlin-1-W612A non β1-integrin binding mutant were re-expressed in KS keratinocytes to see if they could rescue levels of EGFR. Kindlin-1 was tagged with mCherry to enable localisation to be assessed. mCherry alone controls were generated to ensure that any changes in protein levels were not due to overexpression of the mCherry protein. Stable cell lines were generated using lentiviral vectors expressing WT kindlin-1 or kindlin-1-W612A mutant. Cells were lysed and used for immunoblotting (Figure 4.11 (a)). Levels of EGFR were significantly restored in both KS keratinocytes expressing the WT kindlin-1 and kindlin-1-W612A mutant suggesting that the loss of EGFR was due to the loss of kindlin-1 expression in these cells. However, the EGFR levels in the W612A re-expressing cells remained significantly lower than in WT cells (Figure 4.11 (b)).

To determine whether expression of kindlin-1 in KS keratinocytes leads to changes in EGFR localisation, fixed cells were stained for EGFR and analysed by confocal microscopy. In Figure 4.12 KS keratinocytes expressing mCherry alone control vector show mainly perinuclear localisation of EGFR, as seen before. The wild type and kindlin-1-W612A expressing cells showed EGFR localised to the plasma membrane and reduced levels of perinuclear staining. Thus, the expression of kindlin-1 is able to restore normal recruitment of EGFR to the membrane.

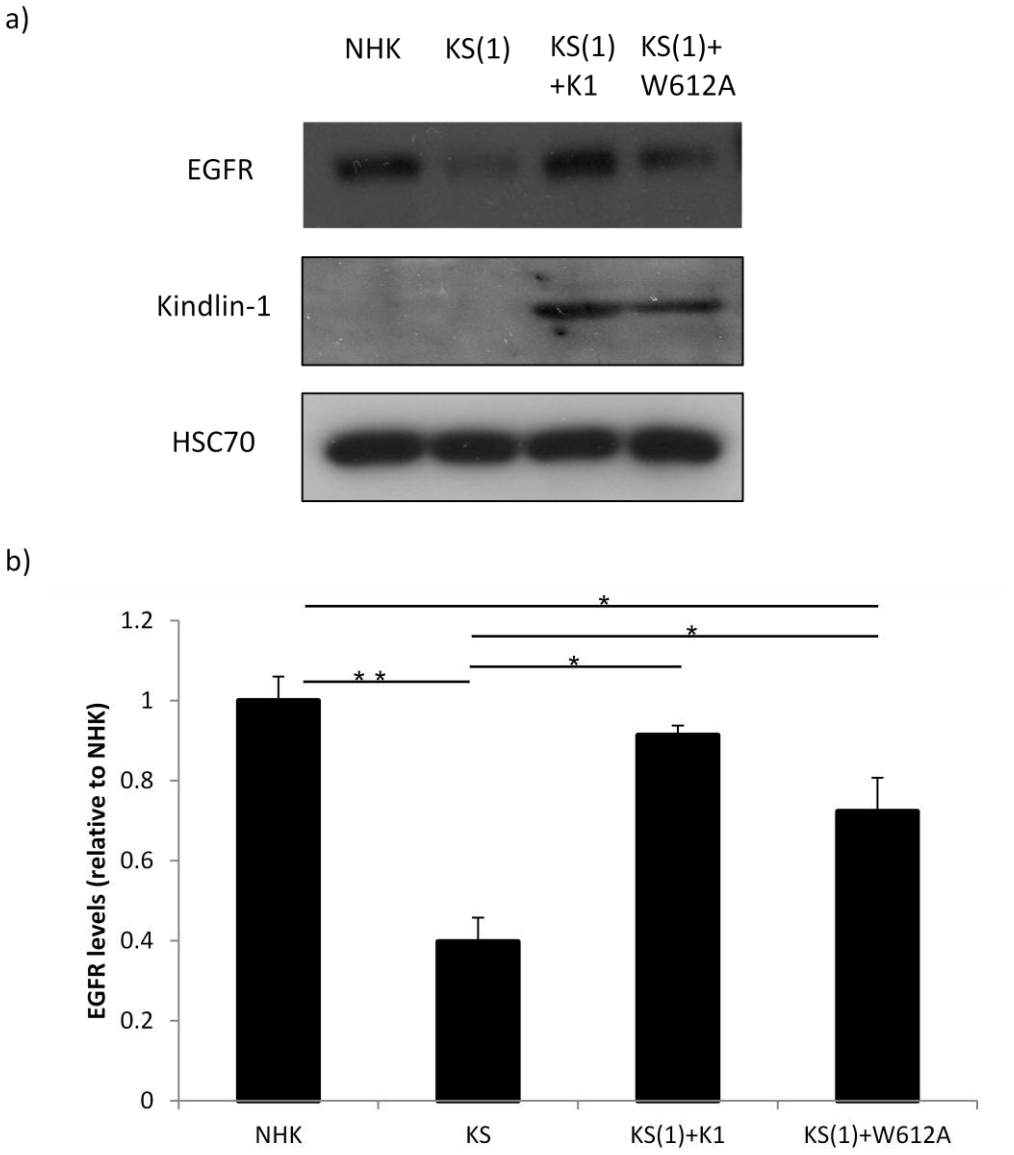


Figure 4.11: EGFR levels are rescued by expressing kindlin-1 in KS keratinocytes.

a) Lysates of KS cells stably expressing mCherry-WT kindlin-1 or mCherry-kindlin-1-W612A mutant and probed for EGFR. b) Graph shows densitometry analysis of EGFR protein levels. Error bars are S.E.M., * = P< 0.05, * * = P< 0.001.

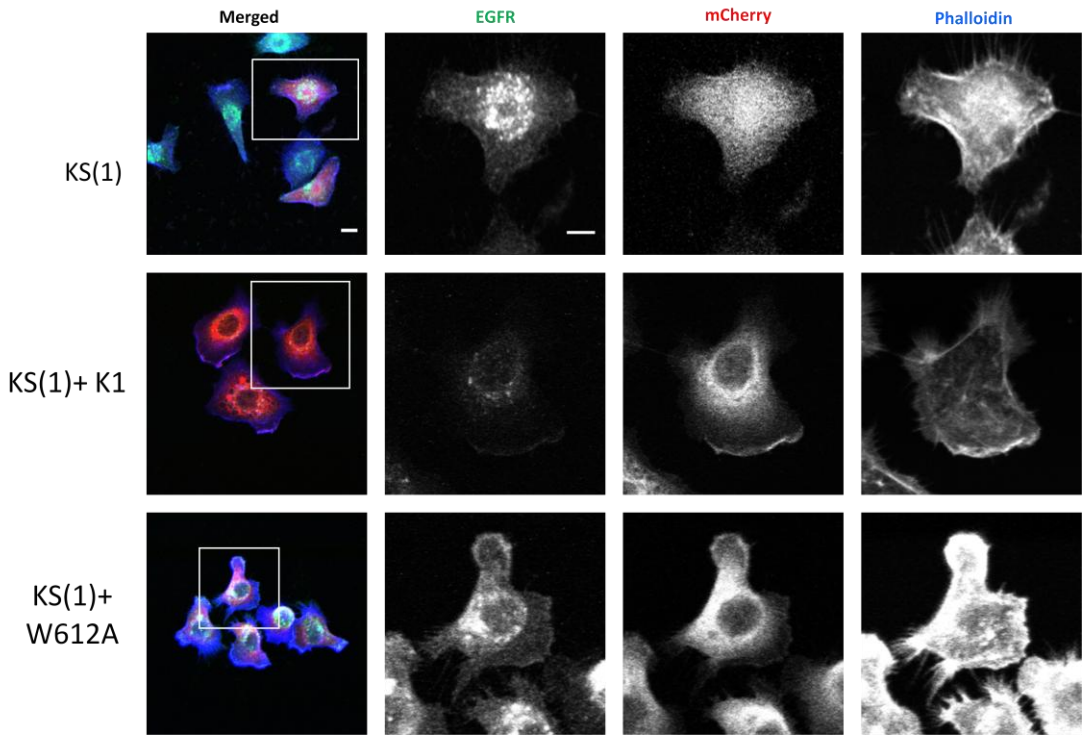


Figure 4.12: EGFR localisation is rescued by kindlin-1 re-expression in KS keratinocytes.

Confocal images of fixed KS keratinocytes expressing mCherry, mcherry-kindlin-1 and mCherry-W612A kindlin-1. EGFR is shown in green, mCherry in red and F-actin in blue (Phalloiden 633). EGFR, mCherry and phalloidin channels have been zoomed in from the original image as highlighted by the white boxes. Scale bar = 10 µm.

The ability of WT kindlin-1 and kindlin-1-W612A to rescue keratinocyte phenotypes was further examined. Cells were fixed and stained for EGFR and total protein intensity and the surface levels of EGFR were analysed as before using confocal microscopy. These measurements were made in cells before and after treatment with EGF. The intensity of EGFR was quantified from immunofluorescence staining of EGFR in cells expressing WT kindlin-1 or kindlin-1-W612A and expressed as a percentage of EGFR levels in NHK control. Representative confocal images are presented in Figure 4.13 (a). The data shows that total and surface levels of EGFR were rescued in KS keratinocytes expressing either WT kindlin-1 or kindlin-1-W612A mutant. The total level of EGFR in these kindin-1 expressing cells did not change after stimulation with EGF which is consistent with that seen in NHK control cells (Figure 4.13 (b)). The surface level of EGFR decreased in response to EGF stimulation in a similar way to that seen in NHK control cells (Figure 4.13 (b)). In cells expressing wild type kindlin-1 the percentage of surface level after EGF stimulation was reduced by more than half (61%), whereas in kindlin-1-W612A mutant expressing cells the surface EGFR levels following EGF was significantly lower than WT kindlin-1 expressing cells (38%) indicating kindlin-1-integrin binding is important for this response.

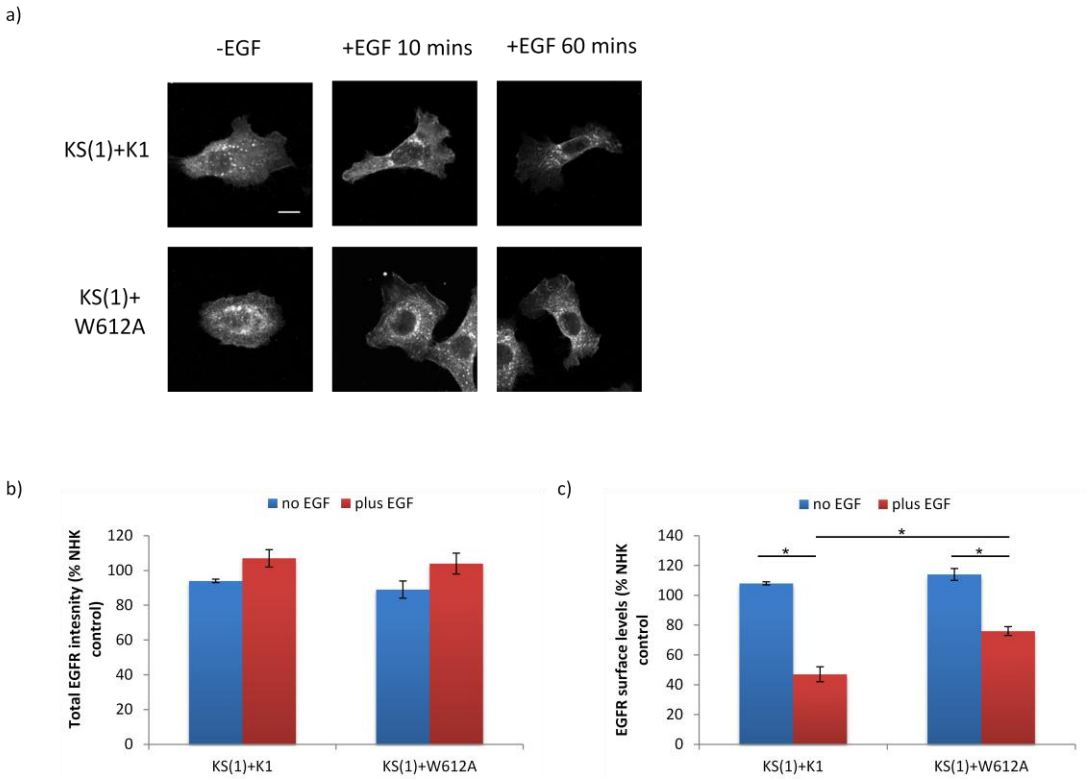


Figure 4.13: Kindlin-1 is able to rescue EGFR surface levels and intensity.

a) Example confocal images of KS cells stably expressing Kindlin-1-mCherry or Kindlin-1W612A-mCherry treated with EGF, fixed and stained for EGFR. Graphs show quantification of EGFR b) total intensity or c) surface levels analysed from >20 images per condition. Error bars are S.E.M., * = P< 0.01 KS (expressing WT kindlin-1) vs no EGF, * = P< 0.05 KS (expressing kindlin-1-W612A) vs no EGF and vs KS (expressing WT kindlin-1) (plus EGF).

To help determine whether loss of directed migration in response to EGF is kindlin-1 dependent, the migration speed and persistence of KS keratinocytes expressing either WT kindlin-1 or kindlin-1-W612A was analysed, as seen in Figure 4.8. Figure 4.14 (a) illustrates examples of the migration tracks of the cells that were analysed. Both migration speed and persistence in kindlin-1 rescued cells were reduced to the same levels as NHK control cells and there was a significant increase in both parameters in response to EGF. The quantification for this data can be seen in Figure 4.14 (b, c). However, cells expressing kindlin-1-W612A did not show restoration of migration speed or persistence to levels seen in either NHK or KS keratinocytes re-expressing WT kindlin-1.

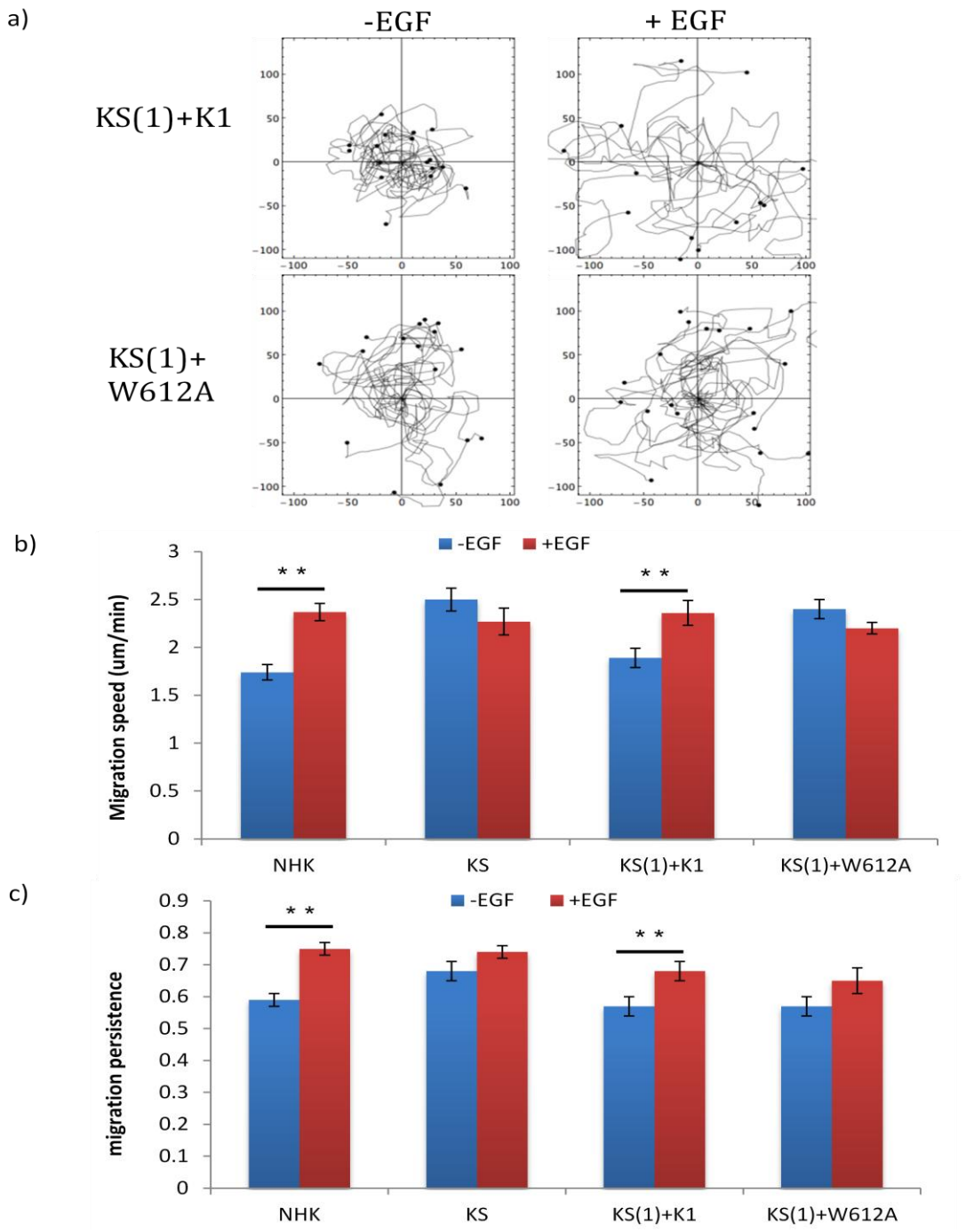


Figure 4.14: Kindlin-1 expression in KS keratinocytes can rescue migration speed and persistence in response to EGF stimualtion.

a) Representative tracks of NHK, KS, KS(1)+K1 (expressing WT kindlin-1) or KS(1)+W612A (expressing kindlin-1-W612A) keratinocytes undergoing migration before or after EGF stimulation (100ng/ml). Graphs of quantification from >60 cells of a) migration speed or b) persistence. Error bars are S.E.M., * * = P< 0.001.

I next aimed to examine whether kindlin-1 may regulate EGFR through formation of a complex. Colocalisation studies of EGFR and kindlin-1 were performed in the presence and absence of EGF to examine whether these proteins show colocalisation and if there is any changes to this localisation in response to EGF stimulation. Representative confocal images are presented in Figure 4.15 (a) from which Pearson's colocalisation coefficient analysis was conducted as before. Analysis revealed in Figure 4.15 (b) indicates that EGFR shows some degree of colocalisation with both with the WT kindlin-1 and the kindlin-1-W612A mutant. After 15 min of EGF stimulation this colocalisation increased significantly. Following further 60 min stimulation, the level of colocalisation was reduced with the WT kindlin-1 but significantly less in the kindlin-1-W612A mutant. This suggests that there is a potential complex formed between EGFR and kindlin-1 and that the interaction may be increased during EGF-dependent cellular responses. Furthermore, the dissociation of the complex requires kindlin-1-integrin binding.

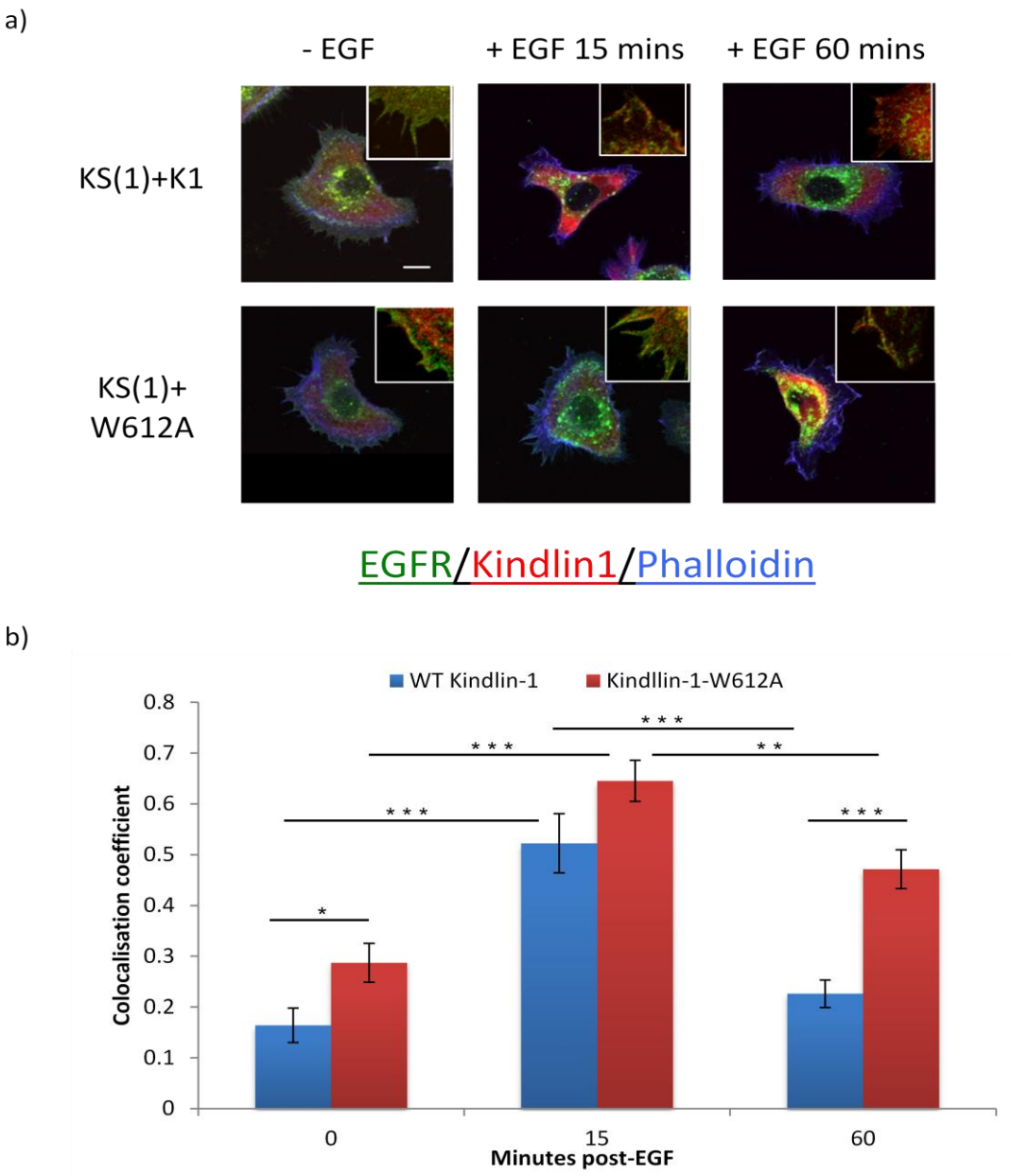


Figure 4.15: Kindlin-1 colocalises with EGFR and colocalisation increases after EGF stimulation.

a) Representative confocal images of KS cells stably expressing mCherry-WT kindlin-1 (KS(1)+K1) or mCherry- kindlin-1-W612A (KS(1)+W612A) (red) with or without EGF treatment, fixed and stained for EGFR shown in green and F-actin in blue (Phalloidin 633). Images were analysed for colocalisation using Pearson's correlation co-efficient analysis. Scale bar = 10 μm. b) % colocalisation shown in graph, Error bars are S.E.M., * = P< 0.05, * * = P< 0.005, * * * = P< 0.0001.

4.2.5 Kindlin-1 interacts with EGFR

From the data collected so far it appeared that EGFR may potentially be an interacting partner of kindlin-1. To explore this hypothesis, GST pulldown experiments were carried out using GST tagged kindlin-1. A GST tagged 9 bp deletion mutant of kindlin-1 was also used for this experiment, as this mutant represents a unique mutation in a KS patient which is located within the F3 subdomian of kindlin-1 and is predicted to be important in integrin binding. Pulldowns were performed with lysates from NHK control cells followed by immunoblotting. Data showed that EGFR is able to form a complex with kindlin-1 and there was no difference in the levels of interaction between WT kindlin-1 and kindlin-1 9 bp deletion mutant (Figure 4.16 (a)). The interaction was quantified relative to GST alone control as demonstrated in Figure 4.16 (b).

To validate the interaction between kindlin-1 and EGFR, and determine whether the interaction was direct, fluorescence resonance energy transfer (FRET) was analysed between EGFR-GFP and kindlin1-mCherry using fluorescence lifetime imaging microscopy (FLIM). FRET is the transfer of energy between two fluorophores in close proximity (less than 10 nm), in this case GFP-EGFR and mCherry-WT kindlin-1 or mCherry-kindlin-1-W612A mutant. The efficiency of FRET was measured using FLIM which determines the change in the fluorescence lifetime of the donor molecule (GFP); if two proteins are interacting, the lifetime will decrease. The example images from the FRET analysis in Figure 4.17 (a) shows the GFP channel (the donor) of EGFR, the mCherry channel (acceptor) of kindlin-1 and also the lifetime map of GFP lifetime represented as a pseudocolour scale. The red colour in the map indicated when FRET was occurring (low lifetime) and blue areas indicated no FRET (higher lifetimes). The FRET efficiency was calculated in multiple cells,

pooled and plotted as a percentage as depicted in Figure 4.17 (b) under conditions of 0 min, 10 min and 60 min EGF stimulation. The FRET analysis confirmed that EGFR is able to interact with kindlin-1. At 0 min there was higher FRET efficiency with the kindlin-1-W612A than the wild type kindlin-1. After 10 min of EGF stimulation the FRET efficiency more than doubles for both wild type (4% to 9.3%) and W612A kindlin-1 (7.1% to 14.9%) implying an increased level of interaction between EGFR and kindlin-1. Following 60 min of EGF stimulation the FRET efficiency decreased to 5.8% for the WT kindlin-1 and a smaller reduction to 12.1% for knidlin-1-W612A mutant. The data suggest that there is preferential binding of EGFR to kindlin-1-W612A mutant and that optimal binding occurs after 10 min EGF stimulation.

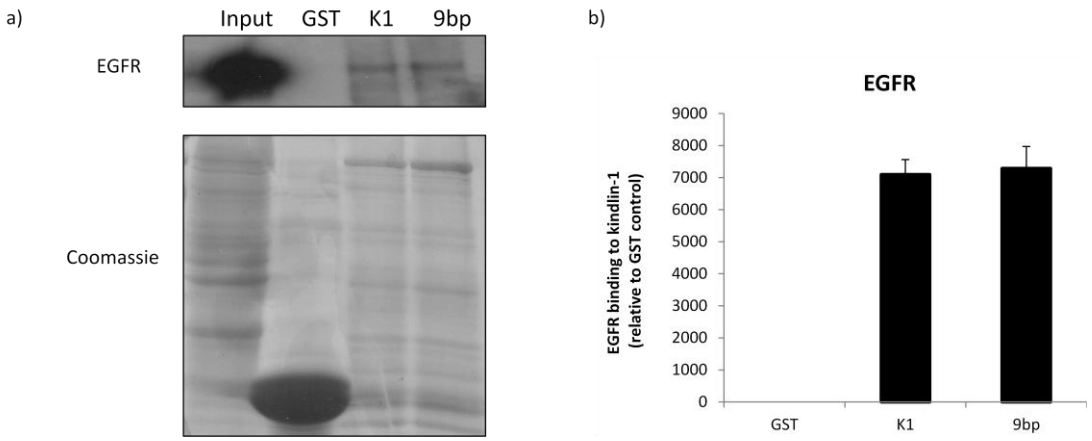


Figure 4.16: Kindlin-1 interacts with EGFR

a) Western blot of pulldown with GST alone, GST-WT kindlin-1- and GST-kindlin-1 9 bp deletion mutant from NHK lysate and probed with anti-EGFR antibody. Coomassie stained gels shows GST-tagged protein used for pulldown assay. b) Graph shows quantification of interaction of EGFR with GST-WT kindlin-1 or GST-kindlin-1 9 bp deletion mutant relative to GST alone control. Error bars are S.E.M.

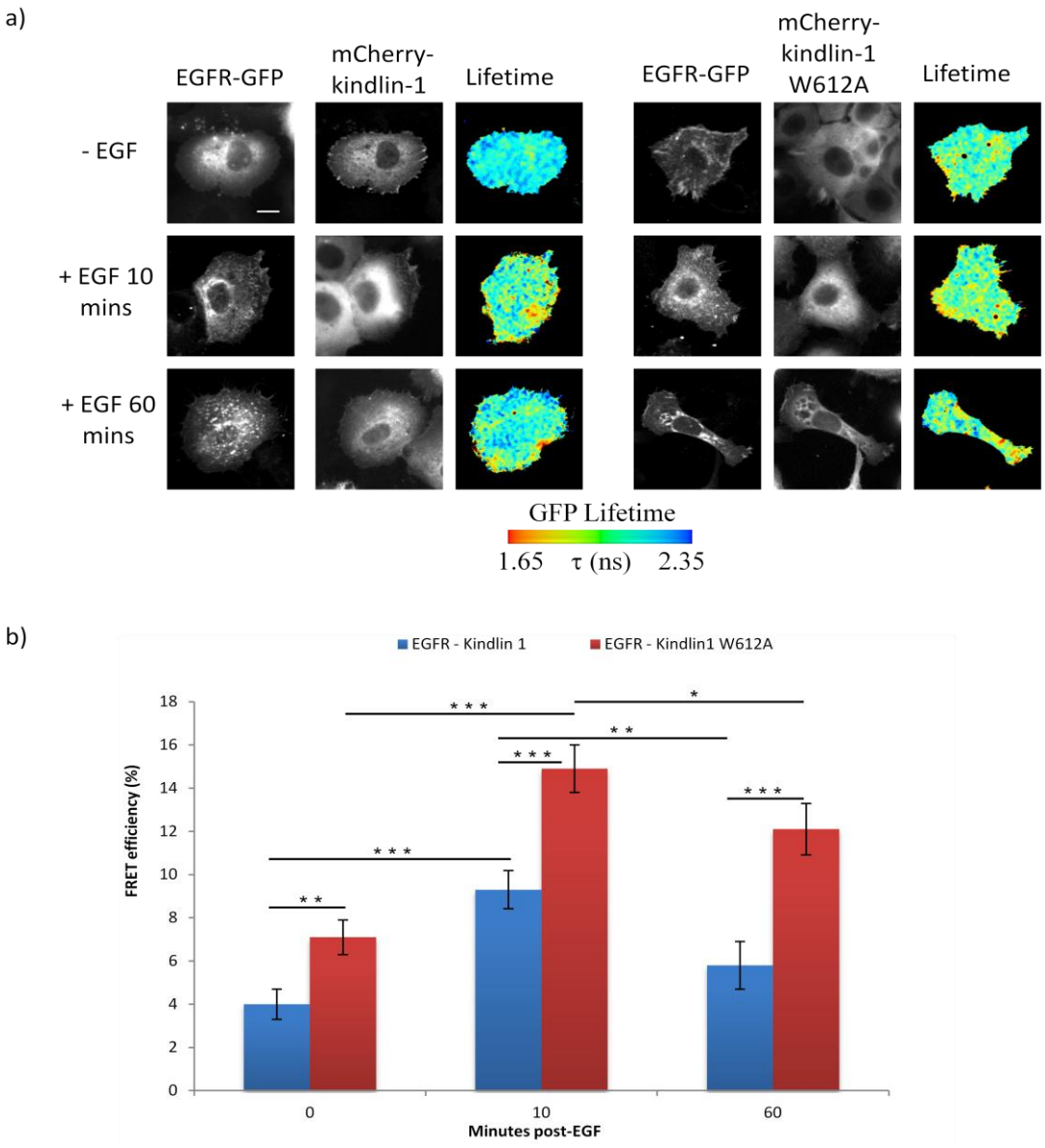


Figure 4.17: FRET analysis validates interaction between kindlin-1 and EGFR.

FRET/FLIM analysis of interactions between EGFR-GFP and WT Kindlin-1-mCherry or W612A kindlin-1-mCherry expressed in KS cells. a) Example images of GFP intensity channel, widefield image of mCherry acceptor and lifetime map of GFP lifetime shown as pseudocolour scale. Red area show low lifetime (FRET), blue is higher lifetimes (no FRET). Scale bar = 10 µm. b) Graph shows cumulative FRET efficiency data from >15 cells per treatment. Error bars are S.E.M., * = P< 0.05, * * = P< 0.005, * * * = P< 0.0001. FRET/FLIM experiment and analysis was done by Dr Maddy Parsons.

4.3 Discussion

The aim of this chapter was to analyse the role of kindlin-1 in the regulation of the receptor tyrosine kinase EGFR, as this protein was found to be reduced in KS keratinocytes through mass spectrometry analysis (Chapter 3). This was then further confirmed by western blotting and FACS analysis, which showed reduction in total and surface levels of EGFR, although there was no overall reduction at gene level. This indicates that protein is being produced normally in the cells but is subsequently degraded. Analysis of EGFR in normal and KS skin showed that EGFR was markedly reduced in the basal keratinocytes, the non differentiated cells, of KS skin, while all the differentiated cells above the basal layer have normal levels of EGFR. This suggests that basal cells routinely have EGFR, however this level is significantly reduced as a consequence of loss of kindlin-1. There are a few possibilities for why this occurs one explanation is that there are compensatory mechanisms in the differentiated cells. Cells undergoing differentiation go through transcriptional changes, for example in the skin integrins are downregulated and keratins are upregulated as the cells move suprabasally (Eckert and Rorke, 1989; Jones and Watt, 1993). Therefore, it is possible that proteins newly expressed in the differentiated cells compensate for the loss of kindlin-1. Another possibility of the difference of EGFR expression in different epidermal layers is that kindlin-1, which has been shown to be expressed higher in basal layers (Lai-Cheong *et al.*, 2009a), is more important in these cells for regulating EGFR than in the upper layers. A change in EGFR localisation was also observed in KS cells, with the receptor being more highly localised to lysosomal compartments suggesting that EGFR is less efficiently recruited to, or maintained at, the plasma membrane to carry out its normal ligand-binding function.

Further characterisation of EGFR signalling responses and the effects of EGFR downregulation in KS keratinocytes revealed functional roles for these changes. NHK cells treated with EGF (the main ligand of EGFR1 in keratinocytes) led to increased internalisation of EGFR, as detected by a significant reduction in surface levels. However, in KS keratinocytes the response was not as great as that seen in NHK. Immunoprecipitation assay showed that EGFR was still being phosphorylated, although at a reduced level due to total reduced levels of EGFR. This suggests that kindlin-1 is not involved in ligand binding or autophosphorylation of EGFR, the first steps that occur during signalling. However, there were clear defects in activation of downstream intracellular signalling, specifically in the MAPK pathway in KS cells following EGF stimulation. This reduced intracellular signalling may be explained by the reduction in EGFR levels in KS cells. Alternatively, it is possible that kindlin-1 is required for initiation of specific signalling steps downstream of EGFR activation. Erk1/2 activation and phosphorylation require recruitment of a host of adaptor proteins and kinases to the EGFR including Grb2, Sos, and the GTPases Ras and Raf. Grb2 and Sos bind directly to the EGFR cytoplasmic domain following tyrosine phosphorylation. Given that loss of kindlin-1 does not inhibit the EGF-induced tyrosine phosphorylation of EGFR, it would seem unlikely that this step in the cascade would be inhibited in KS cells. However, this would be important to determine in future studies.

Functional studies were performed to analyse the behaviour of KS keratinocytes to EGF stimulation. Live imaging showed that after treatment with EGF, NHK cells rapidly contracted, showing large retraction fibres at the plasma membrane and then the cells rapidly start to spread out again after 10 minutes. KS keratinocytes, however, displayed a reduction in this initial contraction and there was an additional

delay in re-spreading after treatment. These data are consistent with previous reports demonstrating spreading defects in KS keratinocytes following re-plating on integrin ligands (Has *et al.*, 2008). Furthermore, there was altered response in migration speed and persistence of KS keratinocytes. Firstly, KS cells showed significantly higher migration speeds than NHK, and NHK showed a significant increase in speed and persistence after EGF stimulation that was not seen in KS keratinocytes. The increased migration speed and persistence before EGF treatment in KS keratinocytes was not expected, as previous studies showed that KS keratinocytes display a reduced migration speed (Herz *et al.*, 2006). An explanation for this inconsistency maybe due to the fact that the data in this thesis was collected by monitoring random migration, whereas other reports have analysed cell migration with *in vitro* wound closure or 'scratch' assays. Thus KS keratinocytes show delayed ability to close an *in vitro* scratch wound but as single cells, migrate faster. The biggest difference between these two assays lies in the fact that scratch wound assays rely on the use of cells as a monolayer, that have formed close cell-cell adhesive contacts, whereas single cells are analysed in random migration assays. Epithelial cell movement as a monolayer into scratch wounds requires collective communication and migration between cells. KS cells are known to have defective cell-cell adhesion both *in vivo* and *in vitro*, and indeed data from this thesis have shown that these cells exhibit reduced E-cadherin levels. This may explain the reason for the reduced migration into wounds, but not as single cells. Indeed as kindlin-1 is known to activate integrins, it seems plausible that the reduced integrin activation in KS cells could result in lower adhesion and thus faster motility. The expression of WT kindlin-1 in KS keratinocytes was able to rescue normal migration speed and persistence and response to EGF in these cells, whereas kindlin-1-W612A was unable to do this.

This also strongly supports kindlin-1:integrin binding as being a critical complex in the control of keratinocyte migration.

The reduction of total EGFR levels was not due to downregulation at gene level but is predicted to be due to increased degradation via the lysosomal compartment in KS keratinocytes. This was validated by showing EGFR levels could be rescued by the lysosomal inhibitor leupeptin and also supported by immunofluorescence studies showing increased localisation of EGFR with LAMP-1 in KS keratinocytes. The data here therefore suggest that kindlin-1 is involved in the recycling of EGFR, and that kindlin-1 potentially acts to retain EGFR at the membrane, or increase EGFR traffic back to the plasma membrane from recycling endosomes. Stable expression of WT kindlin-1 and kindlin-1-W612A in the KS keratinocytes was able to significantly rescue total levels of EGFR and its localisation in the cells, demonstrating a direct link between kindlin-1 and EGFR. Furthermore, surface levels of EGFR and internalisation were also rescued by wild type and W612A kindlin-1 expression. These data also suggest that kindlin-1 regulation of EGFR is not dependent upon kindlin-1-integrin binding. Interestingly, the kindlin-1-W612A non-integrin binding mutant actually led to enhanced retention of kindlin-1 at the plasma membrane following EGF stimulation. This suggests that kindlin-1 that is constitutively non-integrin associated may be more effective at associating with EGFR at the membrane, and potentially cycling of kindlin-1 between integrin and non-integrin compartments plays a key role in allowing kindlin-1 to play a role in EGF signalling. As confocal analysis here demonstrates that kindlin-1 is rapidly lost from adhesions following EGF treatment, and instead localises in membrane ruffles, this would suggest that EGF itself may induce kindlin-1 to cease its localisation with integrins. This may potentially free kindlin-1 up to associate with other binding

partners such as EGFR. Further analysis of the relationship between kindlin-1 and EGFR show that EGFR colocalises with WT kindlin-1 but at an increased level with kindlin-1-W612A. Most interestingly the level of colocalisation of kindlin-1-W612A does not reduce to levels similar to WT kindlin-1 further suggesting a preferential association function of the non-integrin binding mutant.

The colocalisation and binding studies between kindlin-1 and EGFR infer that they may possibly be interaction partners. Also the presence of a NPXY motif in the cytoplasmic domain of EGFR similar to the kindlin-1 binding motif found in β integrin cytoplasmic domains would seem a likely candidate for the binding site for kindlin-1. GST pulldown assays with purified kindlin-1 implicated EGFR as a potential interaction partner for kindlin-1, although purified EGFR would need to be used to comfirm the interaction is direct biochemically. This assay also showed that binding was not altered with a 9 bp deletion mutant of kindlin-1 within a domain adjacent to the integrin binding site in the F3 subdomain. To validate these findings and confirm the interaction, FRET/FLIM analysis was also used. The FRET efficiency, which was an indicator of interaction between two proteins, verified EGFR and kindlin-1 as binding partners in intact keratinocytes. Moreover, it showed preferential binding of EGFR to kindlin-1-W612A, as hypothesised from the EGFR surface levels analysis following EGF treatment. The interaction seems to be optimal after 15 min of EGF stimulation during which EGFR is internalised into the cells, thus binding occurs mostly in the cytoplasm as opposed to the membrane. This further supports the idea of a non-integrin binding pool of kindlin-1 directly associating with EGFR to retain the receptor at the membrane; this may in turn promote more efficient EGFR signalling.

In this present study, important observations are made about the function of kindlin-1. The ability of kindlin-1 to rescue EGFR from degradation in KS keratinocytes implied that it has a function in EGFR recycling. Kindlin-1 interaction with EGFR may facilitate localisation of EGFR back to the membrane and further degradation via the lysosomal pathway. These effects are enhanced with the use of kindlin-1-W612A supporting the proposal that this function is clearly independent to integrin signalling. These data provide insight into a novel role for kindlin-1 in regulating a growth factor receptor, and provide a potentially exciting new mode of cross-talk between integrins and growth factor receptors.

Chapter 5:

Characterisation of a novel KS mutation in kindlin-1 and consequences for protein binding partners

5.1 Introduction

Previous studies have shown that all of the kindlin protein family members are able to interact with β1 and β3 integrin subunits and that kindlin-3 additionally binds to the β2 integrin subunit (Kloeker *et al.*, 2004; Shi *et al.*, 2007; Moser *et al.*, 2009a). Integrins are heterodimeric integral membrane receptors consisting of α and β subunits. Integrin activation and subsequent downstream signalling has been proposed to occur via two distinct mechanisms. Firstly, "outside-in" signalling whereby the ligands that bind to the extracellular domain of integrin cause a conformational change that leads to subsequent changes within the cell. Secondly "inside-out" signalling has been proposed as a mechanism by which binding of intracellular adaptor proteins to the β integrin cytoplasmic tail can lead to subsequent changes in the conformation and affinity of the integrin receptor for its ligands (Harburger and Calderwood, 2009). Kindlin-1 has been proposed to act in concert with talin to regulate integrin activation by changing it affinity to ligand binding (Moser *et al.*, 2009b). Talin consists of a N-terminal globular head domain and a C-terminal rod domain. The head domain comprises the FERM domain, homologous to that found in kindlins (Calderwood, 2004). The F3-PTB like domain of talin required for integrin binding is autoinhibited by the rod domain, and upon calpain cleavage or binding to phosphatidylinositol 4, 5-bisphosphate (PIP2), F3-PTB is exposed to interact with β integrin subunit tail and induce activation (Martel *et al.*, 2001; Yan *et al.*, 2001). The attachment of talin is proposed to lead to the separation of α and β subunits cytoplasmic domains resulting in activation. Similarly, kindlins also bind to the β integrin cytoplasmic tail but at the membrane distal NxxY motif leading to an as yet unknown conformational change in the receptor (Kloeker *et al.*, 2004). The F3 subdomains of talin and kindlin-1 are key to binding the cytoplasmic tail of β

integrin (Calderwood *et al.*, 2002; Shi *et al.*, 2007) and it is hypothesised that kindlin-1 is recruited to the β integrin tail by its PH domain which may bind to a phosphoinositide, another possibility is recruitment through phosphorylation (Moser *et al.*, 2009b). Forty six different mutations in the *KIND1* gene have been identified in KS patients to date (Has *et al.*, 2011a). A number of these result in mutated or truncated protein being produced that has a clear effect on kindlin-1 function, but the specific domains within kindlin-1 that contribute to this function remain unexplored. Moreover, it is currently unclear how talin and kindlin-1 cooperate to activate integrin, but there are three possibilities which have been suggested. First, talin and kindlin-1 bind successively to one β integrin cytoplasmic tail and cause change in ligand binding affinity. Secondly, both proteins may bind at the same time and provide the same effects. Thirdly, talin and kindlin-1 may bind to different β integrin tails resulting in the clustering of integrins and cross-talk between the two proteins (Moser *et al.*, 2009b). Talin and kindlin have not yet been shown to interact with each other, but studies show that they both interact with the β integrin tail at the same time (Bledzka *et al.*, 2012). Further investigation is needed to fully explain how the mechanism of integrin activation is achieved.

Kindlin-1 is also able to form a complex with migfilin, a LIM-containing focal adhesion protein (Lai-Cheong *et al.*, 2008). Migfilin can bind to filamin which in turn binds to actin, therefore providing an indirect interaction between kindlin-1 and the actin cytoskeleton (Lai-Cheong *et al.*, 2008; Malinin *et al.*, 2010). Migfilin is proposed to be a promoter of integrin activation as it binds to the same region in filamin where integrin β cytoplasmic tails bind (Ithychanda *et al.*, 2009) and has been shown to rescue activation of integrin that was inhibited by filamin (Das *et al.*, 2011). Another binding partner of kindlin-1 is kindlin-2, although the functional

relevance of this interaction has yet to be elucidated (Lai-Cheong *et al.*, 2008). FAK and α-actinin have also been shown to interact with kindlin-1, although in depth analysis has not been done to further explore the link between these proteins (Has *et al.*, 2009). These interactions might be involved in the mechanisms by which kindlin-1 conveys outside-in signals and links integrins to the actin cytoskeleton.

ILK has been shown to interact with kindlins, but so far evidence is exclusive to the kindlin-2 family member (Mackinnon *et al.*, 2002; Tu *et al.*, 2003). ILK is an adaptor protein that also interacts with β integrins after its activation where it serves to interact and recruits other proteins to focal adhesions. This includes kindlin-2, PINCH and the F-actin binding proteins parvin and paxillin. In this way ILK links integrins with the cytoskeleton and growth factor signalling pathways (Wu and Dedhar, 2001). ILK may potentially be a binding partner of kindlin-1 as both kindlin-1 and kindlin-2 appear to share many of the same binding partners.

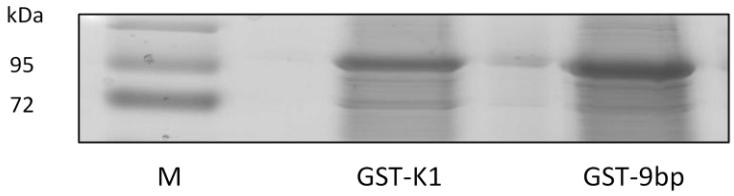
The information about the various binding partners of kindlin-1 provides some insight into its function and to some extent explains the phenotypes observed in KS patients. However, there is a need to investigate other potential interaction partners of kindlin-1 that will help to fully understand the biological role of kindlin-1. The aim of this chapter was to analyse potential new binding partners for kindlin-1 with a view to shedding more light on how kindlin-1 may control keratinocyte function through integrin dependent and independent events. One novel mutation in kindlin-1 within the F3 subdomain, identified in a KS patient, was also employed to help further define the roles of different domains of kindlin-1 in controlling protein complex formation.

5.2 Results

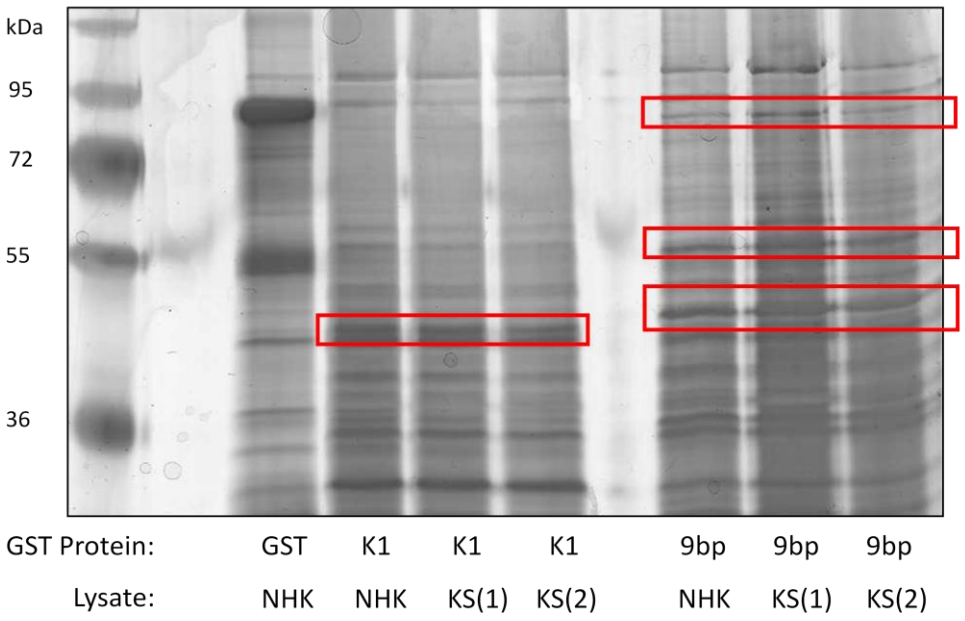
5.2.1 GST pulldown assay and mass spectrometry analysis to identify potential kindlin-1 interaction partners

To investigate one of the initial aims to elucidate novel binding partners of kindlin-1, GST pulldown assays were performed. This required the production of GST tagged WT kindlin-1 and the 9 bp deletion mutant (a novel mutation identified in a single KS patient) purified protein produced in E-coli. The purified proteins were run on a SDS PAGE gel and stained with coomassie to validate the size, integrity and estimate concentration. The purified protein bands can be seen in the example gel in Figure 5.1 (a). The proteins were then used as bait in GST pulldown assays, whereby the proteins were incubated with lysates from NHK or KS keratinocytes. The resulting bound fractions of proteins bound to kindlin-1 were run on a SDS-PAGE gel, silver-stained, and the band profiles between samples compared. The red boxes in figure 5.1 (b) highlight the bands selected for mass spectrometry analysis as these were differentially associated with wild-type versus mutant kindlin-1. The mass spectrometry data revealed a number of interesting target proteins and potential interacting partners including kindlin-2, ILK, peptide sequences within the talin FERM domain, tubulin and ste-20 like kinase, (Figure 5.1 (c)).

a)



b)



c)

Potential Interacting Partners of Kindlin-1
Tubulin (alpha and beta)
Ste-20 like kinase
Kindlin-1
ILK
Talin (FERM)

Figure 5.1: GST pulldown assay followed by mass spectrometry reveals potential interacting partners of kindlin-1

a) 10% coomassie stained acrylamide gel of purified GST tagged WT kindlin-1 and kindlin-1 9 bp deletion mutant b) A silver-stained 12% acrylamide gel showing samples from a pulldown assay using GST–kindlin-1 or GST kindlin-1 9 bp pulldown with NHK and KS keratinocyte lysates. GST alone was used as a negative control. Red boxes highlight protein bands that were analysed with mass spectrometry. c) Table of protein targets identified from the gel bands by mass spectrometry analysis that are potential binding partners of kindlin-1.

5.2.2 Validation of potential interaction partners

In order to validate the results obtained from the mass spectrometry data, the GST pulldowns were repeated but this time used for immunblotting analysis and probed for the specific target proteins (Figure 5.2 (a)). Quantification of the interaction of the target protein with kindlin-1 relative to GST alone from multiple experiments is displayed in Figure 5.2 (b). Previous studies have already shown that kindlin-1 is able to bind to bind to kindlin-2 (Lai-Cheong *et al.*, 2008) and these pulldown experiments further confirm an interaction between kindlin-1 and kindlin-2 although there were no differences in binding between WT and the 9 bp mutant kindlin-1. There was also an interaction seen between kindlin-1 and ILK, in contrast to other reports that had previously only shown an interaction between kindlin-2 and ILK. There was also no change in interaction between ILK and the WT kindlin-1 or the kindlin-1 9 bp mutant. The pulldown experiments also verified an interaction with talin. The anti-talin antibody used here recognises the head domain of talin, and thus the interaction can be detected as being specific to the head domain either alone or present as part of the full length talin. In addition, there was a significant reduction of the head domain of talin binding to the kindlin-1 9 bp mutant compared to WT kindlin-1. Talin has not been previously reported to bind to kindlins so I chose to further analyse this novel interaction in more detail.

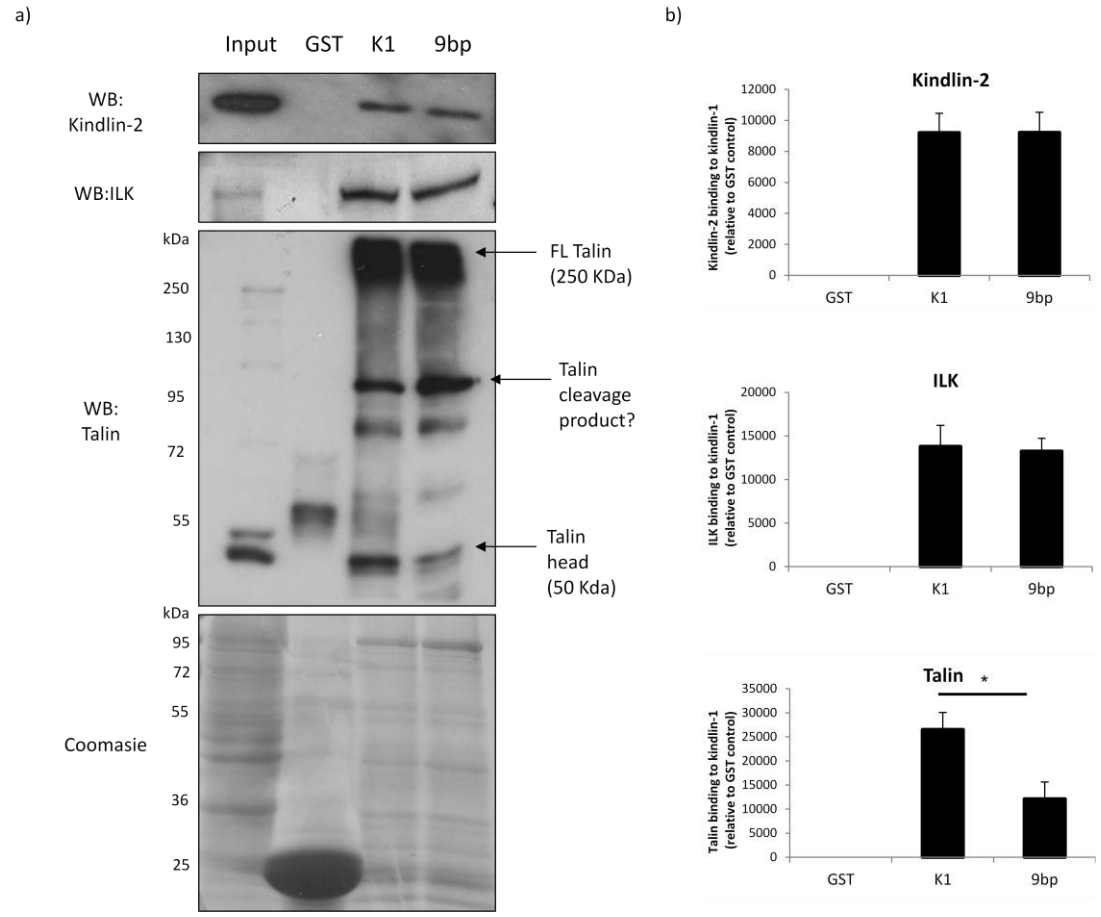


Figure 5.2: GST pulldown followed by immunoblotting validates mass spectrometry results

a) GST pulldown with WT kindlin-1 and kindlin-1 9 bp deletion mutant, analysed using western blotting and probed for kindlin-2, ILK and talin. GST alone was used as a negative control. b) Quantification of GST pulldown assays, with binding relative to GST alone control. Error bars are S.E.M., * = P< 0.05.

5.2.3 Talin is a potential novel binding partner of kindlin-1

Further analysis was required to validate talin as a potential binding partner of kindlin-1 and more specifically to confirm that the binding is specific to the talin head domain. HEK 293T cells overexpressing RFP-talin head (N-terminus FERM domain, residues 1-433) were used to carry out GST pulldown assay with WT kindlin-1 and kindlin-1 9 bp mutant proteins. The western blot analysis displayed in Figure 5.3 clearly shows an interaction between the talin head domain-RFP and kindlin-1. It also shows that the kindlin-1 9 bp mutant results in a lower level of interaction as seen before.

In addition to the pulldown experiments, an immunoprecipitation assay was performed to analyse the interaction between kindlin-1 and talin in keratinocytes. An anti-GFP antibody was used to precipitate out kindlin-1-mCherry expressed in KS cells and its complex of binding partners and resultant complexes were subsequently probed for talin. There was no available anti-mCherry antibody but since it shows a high homology with GFP, the anti-GFP antibody was used to detect the mCherry protein. mCherry alone expressing cells were used as a control for non-specific binding. The immunoprecipitation assay shows further confirmation that the talin head domain does form a complex with kindlin-1 in human keratinocytes (Figure 5.4).

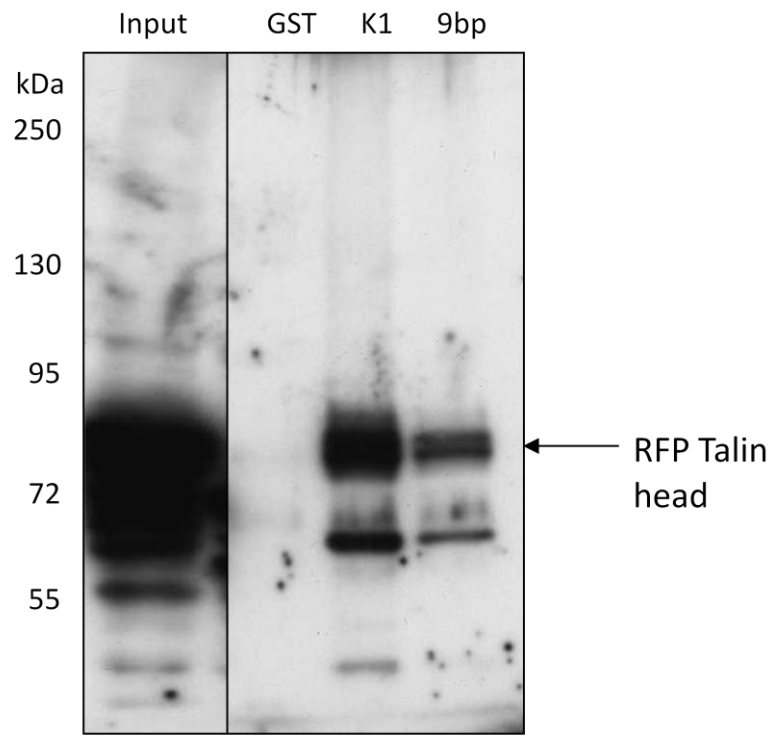


Figure 5.3: Kindlin-1 interacts specifically with the head domain of talin.

Western blot of GST pulldown with GST-WT kindlin-1 or GST-kindlin-1 9 bp and HEK 293T lysates expressing the RFP-head domain and probed with RFP.

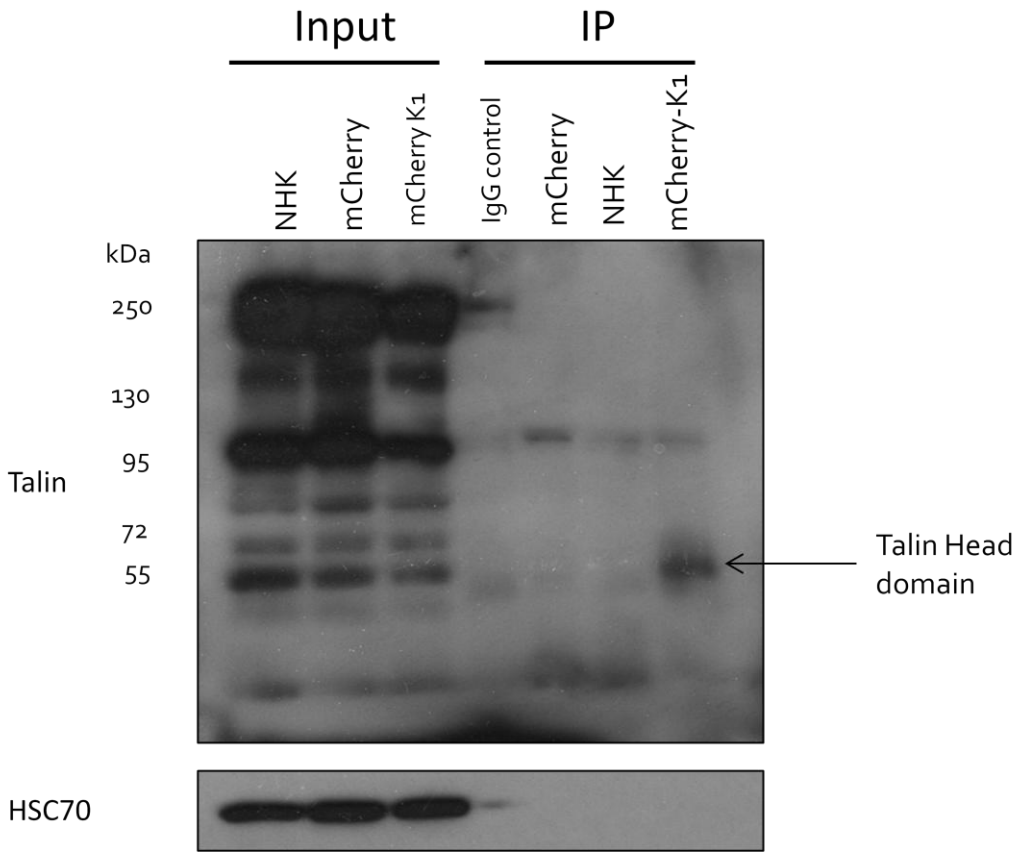


Figure 5.4: Immunoprecipitation of kindlin-1 with talin further validates complex formation.

Western blot of immunoprecipitation assay of kindlin-1 from NHK lysates stably expressing mCherry-kindlin-1 and probed for talin. Rabbit IgG fraction and lysates from untransfected NHK, and NHK expressing mCherry alone were used as controls.

5.2.3 Talin levels are increased in KS keratinocytes

Given the identification of this novel interaction, lysates from KS keratinocytes were analysed to determine whether loss of kindlin-1 resulted in any changes in total levels of talin. Firstly, the expression of the *TLN1* gene was evaluated using semi-quantitative PCR. Analysis revealed increased expression of *TLN1* mRNA in keratinocytes from KS patients compared to controls (Figure 5.5 (a)). Analysis of total protein levels of talin by immunoblotting also demonstrated an increase in expression level of both full length and head domain in KS keratinocytes (Figure 5.5 (b)). Quantification of full length talin (~270 kDa) demonstrated a significant increase in this molecular weight species of ~100% in both KS keratinocyte cell lines (Figure 5.5 (c)).

The localisation of talin was subsequently analysed by immunostaining and confocal microscopy in NHK and KS cells. Example images are shown in Figure 5.6. Talin localised mainly at focal adhesions at the periphery of the NHK cells but was also present in the cytoplasm. This was also seen in KS keratinocytes as there was no change in localisation.

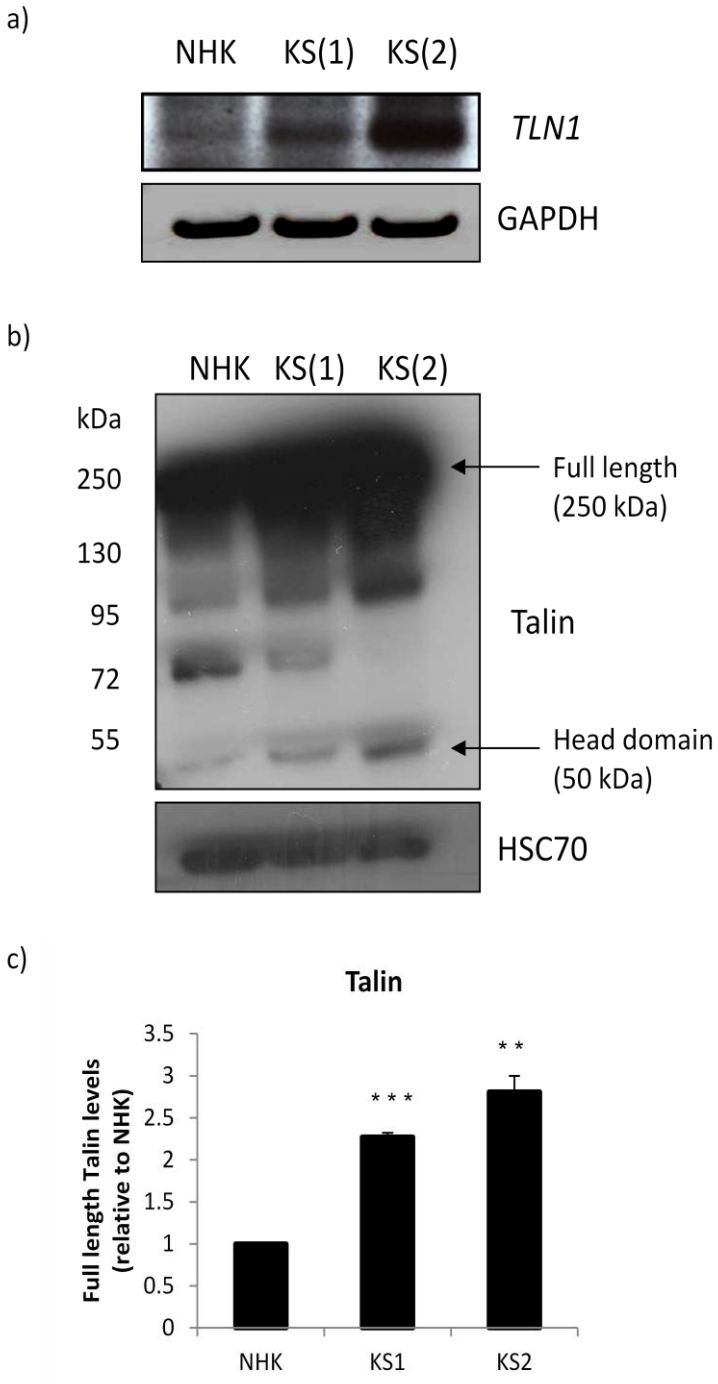


Figure 5.5: Talin expression is increased in KS keratinocytes.

Characterisation of the gene and protein level of talin NHK vs. KS keratinocytes. a) semi-quantitative RT-PCR of *TLN1* mRNA levels in NHK and KS keratinocytes. b) Western blot example of full-length talin total protein level in NHK vs. KS keratinocytes. c) Graphs show densitometry analysis of talin levels. Error bars are S.E.M., n=3, ** = P<0.001, ***= P<0.0001.

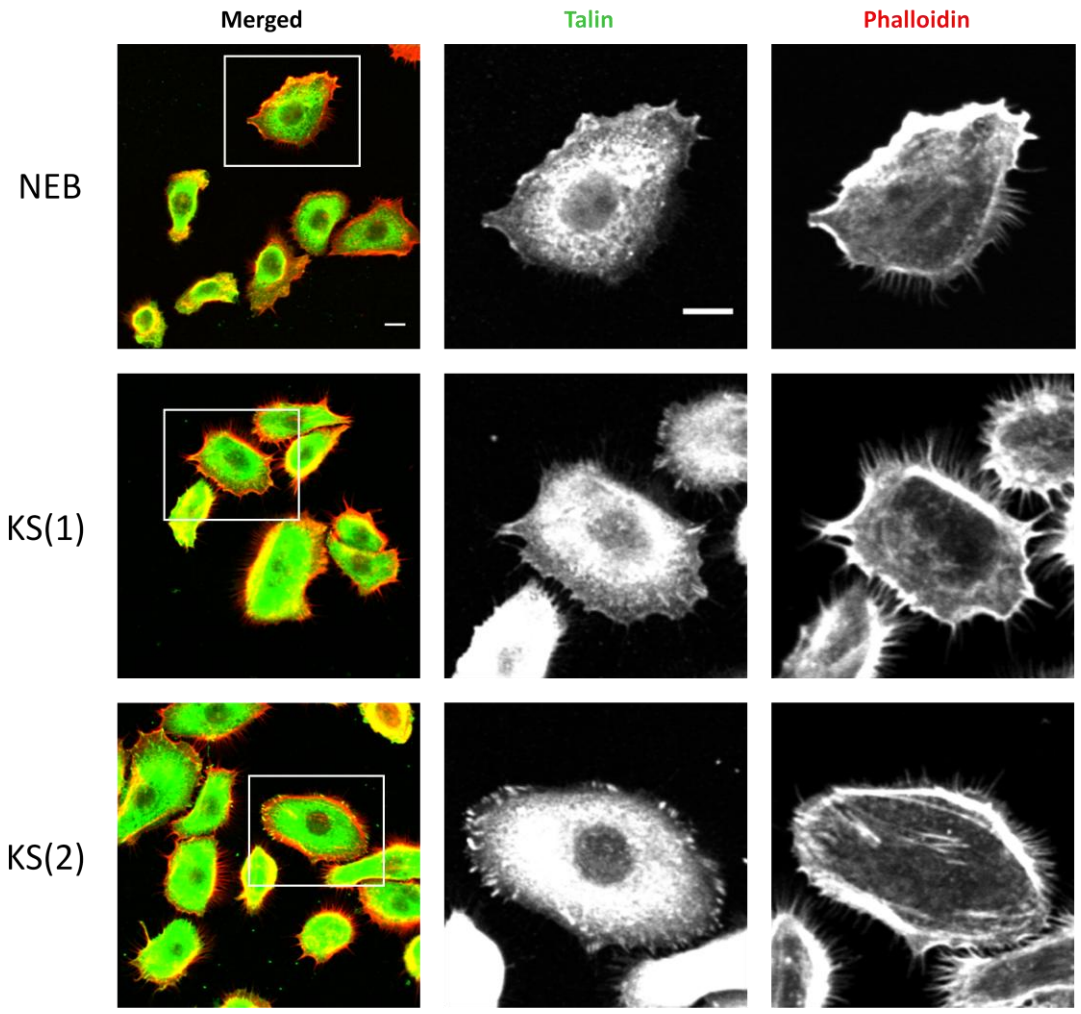


Figure 5.6: Talin localisation does not change in KS keratinocytes.

Confocal images of fixed NHK or KS keratinocytes. Endogenous talin is shown in green and F-actin in red (Phalloidin 633). Talin and phalloidin channels have been zoomed in from the original image as highlighted by the white boxes. Scale bar = 10 µm.

5.2.4 Talin levels in KS keratinocytes are rescued by re-expression of kindlin-1

The generation of KS cells re-expressing mCherry-kindlin-1 (see Chapter 4) allowed for further investigation into the link between talin levels and kindlin-1. KS keratinocytes stably expressing either WT kindlin-1 or kindlin-1-W612A (the non-integrin binding mutant) were analysed for talin levels. Figure 5.7 (a) is an example western blot showing talin levels in NHK and KS keratinocytes alongside KS cells expressing WT kindlin-1 or kindlin-1-W612A. Data demonstrates a significant reduction of talin levels, (Figure 5.7 (b)) suggesting both WT kindlin-1 and kindlin-1-W612A are able to regulate levels of talin.

Furthermore, analysis of KS keratinocytes by confocal microscopy demonstrated that mCherry-WT kindlin-1 colocalised with talin at focal adhesions. However, mCherry-kindlin-1-W612A was not localised at focal adhesions as has been shown previously (Harburger *et al.*, 2009) (Figure 5.8).

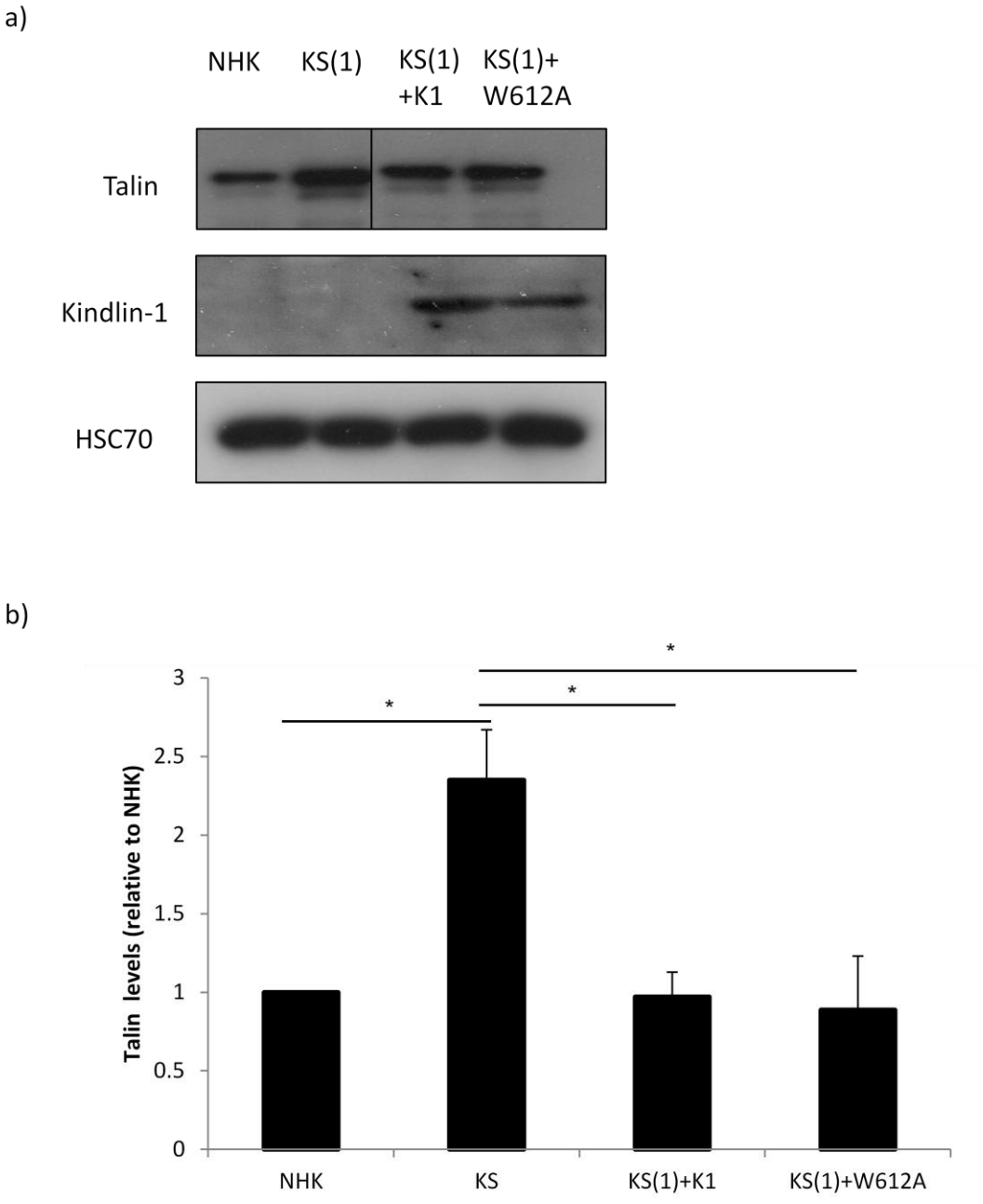


Figure 5.7: Expression of WT kindlin-1 or Kindlin-1-W612A restores talin levels in KS keratinocytes.

a) Western blot analysis of full-length talin total protein levels in NHK or KS keratinocytes stably expressing mCherry alone, mCherry-WT kindlin-1 or mCherry-kindlin-1-W612A mutant. b) Graphs show densitometry analysis of talin levels from three independent experiments. Error bars are S.E.M., n=3, * = $P<0.05$.

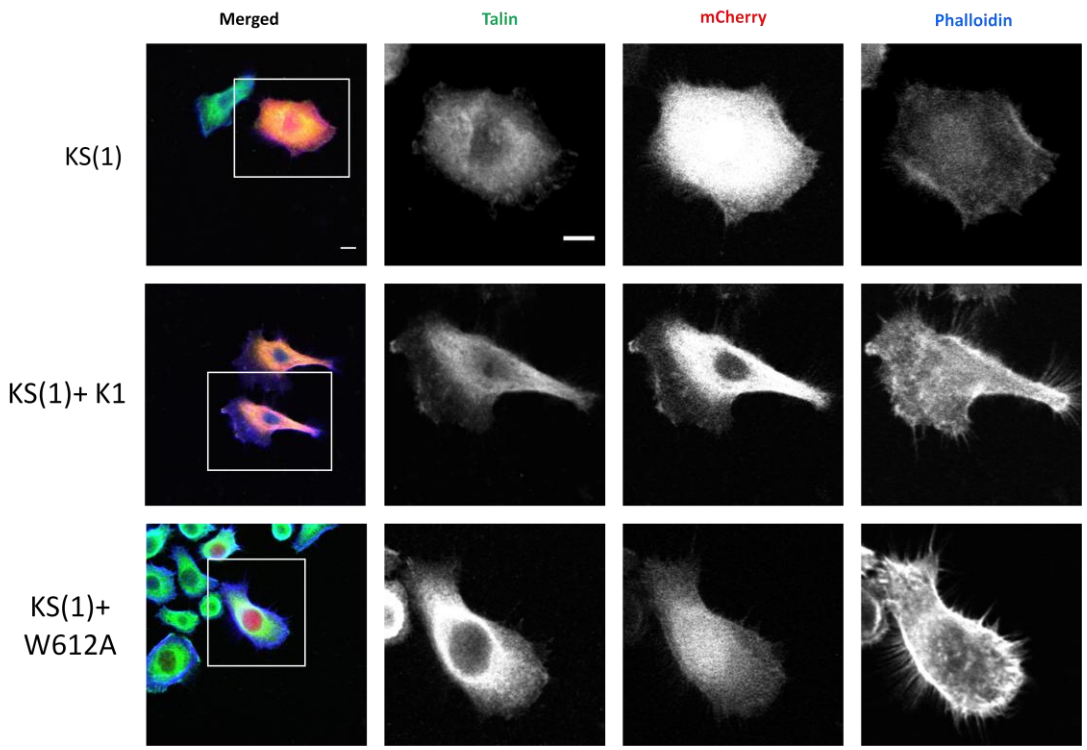


Figure 5.8: Talin localisation rescued by kindlin-1 expression in KS keratinocytes.

Confocal images of fixed KS keratinocytes expressing mCherry alone, mCherry-WT kindlin-1 or mCherry-kindlin-1-W612A. Endogenous talin is shown in green, mCherry in red and F-actin in blue (Phalloidin 633). Talin, mCherry and phalloidin channels have been zoomed in from the original image as highlighted by the white boxes. Scale bar = 10 µm.

5.3 Discussion

In this chapter I have addressed one of the major aims of my thesis: to identify novel binding partners of kindlin-1. Very few binding partners have been reported for kindlin-1 and more information is needed about how this protein is regulated and how it can control integrin-dependent and potentially independent functions.

The GST pulldown technique was employed as it is a useful starting tool for identifying potential protein-protein interactions. GST pulldowns were used as a screening method to identify new interacting proteins for kindlin-1, as well as those proteins that associate differentially with a mutant kindlin-1 identified in a KS patient. Purified GST-tagged WT kindlin-1 and kindlin-1 9 bp mutant were used to precipitate the complex of proteins bound to kindlin-1 from NHK or KS lysates. The aim was to compare band profiles from WT kindlin-1 and the kindlin-1 9 bp mutant protein pulldowns to identify changes in interactions due to the mutation and potentially provide further insight into kindlin-1 function. Mass spectrometry was used to analyse the protein bands that showed differential binding. The resultant peptide coverage of the identified bands revealed a number of interesting target proteins; kindlin-2, ILK, Ste-20 like kinase, tubulin (alpha and beta) and talin (FERM domain). Kindlin-2 has previously been characterised as a binding partner of kindlin-1 (Lai-Cheong *et al.*, 2008) and this study has further validated this interaction. The functional consequence of this interaction is still unclear, but potentially may provide a mechanism by which kindlin family members are able to regulate one another. Other FERM domain proteins are known to undergo self-association (such as talin and ezrin) and potentially dimerisation, and this is thought to restrict access for regulatory and signalling binding partners. In the case of ezrin, this autoinihbition is released through control of a c-terminal phosphorylation site

(T567, through protein kinase C; (Wald *et al.*, 2008)). Whilst the phosphorylation status, and self-association of kindlin proteins in intact cells remains unknown, this mechanism of regulation would be important to study in future to understand how kindlin proteins may co-operate with one another to control cellular function.

ILK has not previously been shown to interact with kindlin-1 but is a known binding partner of kindlin-2 (Montanez *et al.*, 2008). This study described ILK as an interacting partner of kindlin-2 and that ILK recruitment to focal adhesions depends on binding to kindlin-2. Furthermore, ILK has been shown to act as a molecular switch in conjunction with migfilin as a regulator of integrin activation (Ithychanda *et al.*, 2009; Das *et al.*, 2011). GST pulldown analysis in this thesis has established that ILK is also able to interact with kindlin-1, and it is possible that this interaction may have similar function to the interaction between ILK and kindlin-2 in recruitment of ILK to focal adhesions. Given that keratinocytes express both kindlin-1 and -2 it would seem unlikely that ILK is no longer targeted to focal adhesion in KS cells, as kindlin-2 should be sufficient to localise ILK. However, it would be important to analyse the localisation and dynamics of ILK in KS cells in more detail in future to determine whether kindlin-1 plays a specific role in this context, and one that kindlin-2 cannot compensate for. Interestingly, a recent study has highlighted a role for ILK in controlling keratinocyte responses to EGF (Ho and Dagnino, 2012). This study demonstrated that ILK and β1 integrins are required for keratinocytes to establish front-rear polarity in response to EGF in a rac-dependent manner, and that this in turn dictates epithelial cell migration. Given the findings in Chapter 4 of this thesis, demonstrating a potential interaction between EGFR and kindlin-1, this presents an interesting possibility that kindlin-ILK-EGFR complexes

may be required for efficient keratinocyte responses to EGF. This will be an important hypothesis to test in future studies.

Another potential novel binding partner for kindlin-1 identified in this study was talin. This protein was a particularly interesting target as it is a very well characterised focal adhesion protein that contains a N-terminal FERM domain (similar to kindlins), and along with kindlins is also known to play a key role in activation of integrins through inside-out signalling. Further analysis with GST-pulldown and immunoprecipitation assays showed that interaction was specific to the head domain of talin and this interaction was considerably effected with the kindlin- 1 9 bp mutant. These experiments do not, however, confirm that the complex between kindlin and talin is direct and not mediated through an additional third binding partner. In order to analyse this, GST kindlin-1 pulldowns would need to be repeated using recombinant purified fragments of talin. An obvious potential intermediate binding partner would be β1 integrin, as both proteins are known to associate with the cytoplasmic domain of this receptor. However, immunoprecipitating either talin or kindlins with integrins is very difficult in the absence of chemical cross-linkers as the binding is very sensitive to detergent extraction. Integrins would therefore seem to be an unlikely potential molecular bridge to explain the interaction data in this thesis. It is also not clear from the pulldown data presented here whether the complex depends upon the ability of kindlin-1 to bind integrins. The binding site on the integrin cytoplasmic domain for talin and kindlins are distinct so it is possible that the two molecules can occupy the same integrin tail, and potentially bind to one another at the same time. However, another possibility is the interaction between kindlin-1 and talin provides a mechanism to recruit or remove these proteins to and from the plasma membrane. It

will be important to test direct interactions and potential mediators of this complex formation in future studies.

Further investigation into the levels of talin in KS keratinocytes showed that mRNA and total protein levels were significantly upregulated in these cells. This was also supported by immunofluorescence analysis, which showed increased intensity of talin staining in KS cells. Expression of both WT kindlin-1 and kindlin-1-W612A in KS cells was able to significantly reduce talin levels back to those seen in NHK. These data raise the possibility that talin is being upregulated by KS cells to compensate for the lack of kindlin-1. This would be particularly important in the context of integrin activation. However, increased talin levels cannot make up for the loss of kindlin-1 as integrin activation in KS cells is significantly lower than that in NHK (Lai-Cheong *et al.*, 2009a). This further supports the concept that both talin and kindlins are required for co-operative induction of maximum integrin activity. Moreover, re-expression of kindlin-1-W612A resulted in rescue of normal talin levels, suggesting that kindlin-1 has an important role outside of the association with integrins in controlling talin levels. Indeed, kindlin-1 may play a more direct role in the regulation of talin transcription, potentially through regulating specific transcription factors. Given the fact that the GST pulldown data in this thesis also suggests talin and kindlins form a complex, this association may also act to regulate levels of talin via a negative feedback mechanism. The discovery of this interaction adds a new dynamic to integrin activation and regulation and the roles that talin and kindlin-1 play in this process.

Chapter 6: General discussion and conclusions

6.1 Kindlin-1 regulates protein levels

6.1.1 Proteomic analysis of KS keratinocytes

The data in this thesis have identified a number of proteins that showed reduced expression in patient-derived KS keratinocytes. The differential expression of these proteins, which include E-cadherin, TSP-1 and EGFR, were initially detected using silver staining of whole cell lysates, followed by mass spectrometry and subsequently validated by western blotting. Mass spectrometry is a powerful analytical technique that is based on the detection of the molecular mass of a molecule. The method works by generating charged particles by ionising the chemical compound and then measuring the mass to charge ratio. This information then permits the mass of particles to be calculated and subsequently the elemental composition of a molecule can be determined. Many studies utilise this technique to identify differentially regulated proteins, novel interaction partners, and downstream molecules of a particular pathway. An example of this includes a study which used a co-immunoprecipitation assay followed my mass spectrometry to identify interaction partners for the N-terminal region of periplakin (a cytoskeletal linker protein), with identification of plectin as a target protein (Boczonadi *et al.*, 2007). Another example is the use of 2D gel electrophoresis followed by mass spectrometry to reveal proteins that play a role in keratinocyte transformation and contribute towards tumourigenesis (Akgül *et al.*, 2009). These examples highlight the value and effectiveness of this technique. Mass spectrometry proved to be a highly useful tool for analysing isolated protein bands in my study, however, there were some limitations associated with it. The technique is a very sensitive one and it detected a number of non-specific proteins in each sample, largely due to the fact that it is difficult to fully resolve the bands excised from the SDS-PAGE gels to isolate single proteins. However, despite

this limitation a number of proteins identified were subsequently shown to be significantly reduced in the KS keratinocytes. Those proteins that were reduced in KS keratinocytes, namely TSP-1 and EGFR, were not downregulated at the gene level, as confirmed by semi-quantitative RT-PCR. This highlights the strength and importance of the application of proteomics methods as these targets would not have been detected if only a transcriptomic or gene chip approach had been taken. Gene chip analysis (or microarrays) is a useful tool to determine the gene expression profiles between different samples; it provides important information about genes as well as pathways that are involved in biological processes. For example, the use of microarray allowed the detection of kindlin-1 as a TGF-β inducible gene (Kloeker *et al.*, 2004). Another example of the use of this technique was in determining the effects of mutant keratin 5 and 14 in EB simplex. This allowed analysis of the changes in expression of other genes to explain some of the underlying mechanisms and other aspects of the EB simplex associated pathology (Liovic *et al.*, 2009). They detected a downregulation of various genes that encode components of the cell junction, which they proposed to indirectly contribute to the blistering and skin fragility in the EB simplex patients (Liovic *et al.*, 2009). However, a drawback of the microarray analysis technique is that strong gene expression resulting in abundant mRNA does not necessarily result in abundant protein expression or activation. Thus, key information about the functional molecules may be missed, which can be complemented by using proteomic methods.

6.1.2 Kindlin regulation of protein levels is integrin-dependent and independent

Both TSP-1 and EGFR were significantly reduced in two KS keratinocyte cells lines in comparison to NHK cells. Furthermore, the localisation of these proteins were also disrupted in skin and cells from KS patients. TSP-1 was retained at the membrane of the KS keratinocyte cells and was not secreted from the cells or deposited in the same pattern as with NHK cells. Re-expressing TSP-1 into KS keratinocytes was unable to rescue the localisation and secretion pattern in these cells indicating that the defect lies not only in the regulation of TSP-1 but perhaps also in its secretory mechanism. However, the introduction of WT kindlin-1 was able to partially rescue TSP-1 levels as well as its deposition and secretion from the cells, thereby providing a link between kindlin-1 and TSP-1 regulation. Interestingly, expression of the kindlin-1-W612A non-β1 integrin binding mutant was unable to rescue TSP-1 expression and deposition in KS keratinocytes, highlighting the importance of integrin-kindlin binding and potentially implicating integrin activation in the process of TSP-1 regulation and function. It would be important to test this relationship by directly activating integrins in KS keratinocytes to test whether this can upregulate TSP-1 expression levels and also its deposition. This could be done by adding exogenous Manganese (Mn), which is able to activate integrins independently of inside-out signalling through the metal-ion binding site within the extracellular domain of the receptor. Alternatively, mutations could be introduced into the β1 integrin subunit which can cause constitutively active or inactive integrins. It would also be interesting to determine whether conditional loss of β1 integrins in keratinocytes, or mutation of the cytoplasmic domain responsible for binding to kindlin-1, results in a similar loss of TSP-1 levels. Previous studies have shown that β1 integrin may regulate TSP-1 expression. For example, the

downregulation of β1 integrin in prostate cancers resulted in a downregulation of TSP-1 (Goel *et al.*, 2009), indicating that β1 integrin downstream effectors may contribute to the TSP-1 pathway. However, the mechanism and the particular signalling molecules involved need further investigation.

Despite the greatly reduced TSP-1 protein levels in KS keratinocytes, TSP-1 mRNA levels were normal compared to NHK controls. This finding raises the possibility that TSP-1 may be degraded in these cells, potentially due to lack of proper secretion and incorporation into the ECM. Further investigation of this possible degradation would require use of proteasomal or lysosomal inhibitors to assess their ability to rescue TSP-1 levels. Any rescue observed would strongly suggest that TSP-1 secretion and degradation is regulated through kindlin-1-mediated integrin activation. The fact that kindlin-1 regulation of TSP-1 appears to be entirely integrin-dependent raises the possibility of a new mechanism through which β1 integrin activation might act to stabilise TSP-1 and thereby protein levels in the skin.

Surface levels of EGFR were also found to be significantly reduced in KS keratinocytes and in the basal cells of KS patient skin. More detailed analysis in cells revealed increased EGFR localisation at the perinuclear region. In addition, KS keratinocytes showed altered responses to EGF stimulation, as EGFR was not internalised and the localisation did not change as expected with prolonged stimulation with EGF, and as seen in NHK cells. Moreover, analysis of downstream signalling molecules of the EGFR transduction pathway showed that ERK phosphorylation was greatly reduced suggesting defective EGFR signalling or activation. There is a possible link between the observed TSP-1 reduction and defects in EGFR signalling in KS patients. Expression of TSP-1 is upregulated in

response to various growth factors including PDGF, EGF, TGF-α, TGF-β and bFGF (Majack *et al.*, 1985; Nickoloff *et al.*, 1988; Penttinen *et al.*, 1988; Donoviel *et al.*, 1990). Studies have shown that TGF-β1 can upregulate levels of TSP-1 through the p38 MAP kinase pathway (Okamoto *et al.*, 2002). Moreover, both EGF and TGF-α were also shown to enhance TSP-1 expression in human keratinocytes. TGF-α, but not EGF, altered expression by changing mRNA stability (Nickoloff *et al.*, 1988). This study suggested that multiple growth factors and ECM components can interact to regulate keratinocyte behaviour in the skin, as the EGF-stimulated keratinocyte proliferation was associated with the induction of TSP-1 biosynthesis (Nickoloff *et al.*, 1988). Therefore, a loss of EGFR signalling, as observed in KS keratinocytes, may contribute to the loss of expression of TSP-1. EGFR appears to be regulated by an altogether different mechanism to that of TSP-1. Both the WT kindlin-1 and kindlin-1 W612A non-β1 integrin binding mutant were able to rescue EGFR total and surface levels and also its response to EGF stimulation. These data suggest that kindlin-1 is able to regulate EGFR in a totally integrin independent manner.

The role of kindlin-1 in E-cadherin regulation was not investigated further in this thesis due to time constraints. However, the potential regulation of E-cadherin by kindlin-1 does offer a potentially interesting new insight into explaining some of the phenotypes seen in KS keratinocytes. E-cadherin is a protein found in adherens junctions that mediates cell-cell adhesion in a calcium-dependent manner. E-cadherin, in particular, plays a critical role in maintaining epithelial cell and tissue structure (Roy and Berx, 2008). As detected by mass spectrometry followed by western blotting (Results Chapter 1 of this thesis), E-cadherin was significantly reduced in both of the KS keratinocyte cell lines. It is possible that the epithelial integrity in KS patients is compromised due to a loss of E-cadherin and consequent

disruption in cell-cell junctions. Previous *in vitro* studies have demonstrated that E-cadherin is involved in controlling cell migration and cell shape (Roy and Berx, 2008). Thus, the loss of E-cadherin may also be a contributing factor in the changes noted in migration and cell shape in KS keratinocytes (Herz *et al.*, 2006; Has *et al.*, 2009). Data in this thesis have also shown defects in cell-cell adhesion in KS keratinocytes that may be explained by the aberrant cell adhesions as well as the reduced migration seen in wound closure assays performed with these cells. Furthermore, there is evidence of cross-talk between cadherins and integrins especially in the processes of cell adhesion, motility and contractility. Cross-talk can occur through different ways, for example, the adhesion of the cell to specific ECM protein or to neighbouring cells can result in a change of expression and levels of particular proteins involved in regulating adhesion (Weber *et al.*, 2011). In addition to this, integrins and cadherins also have convergence points downstream of their signalling pathways, which may include non-receptor tyrosine kinases, adaptors and scaffolding proteins and small GTPases (Weber *et al.*, 2011). There is also some evidence to suggest that GFRs such as insulin-like GF-1 receptor (IGF1R) can facilitate lateral associations of integrins and cadherins thereby mediating a point of interaction between the two receptors (Canonici *et al.*, 2008). This finding raises the possibility of a defective link between integrins, EGFR and E-cadherins leading to the defects seen in KS keratinocytes. A previous study has also shown a reduction in E-cadherin levels in KS keratinocytes, which along with other phenotypes displayed that correlated with an epithelial-to-mesenchymal transition (EMT) (Qu *et al.*, 2012). Kindlin-1 has not been reported to be found in cell-cell junctions although its family member kindlin-2 does localise to cell-cell junctions and may be involved in the formation of these junctions (Ussar *et al.*, 2006; He *et al.*, 2011). It is plausible that

kindlin-1 may transiently localise to cell-cell junctions during certain conditions, but further analysis of this is required. It is also possible that kindlin-1 may regulate E-cadherin through other proteins, perhaps via kindlin-2 which is a binding partner of kindlin-1 (Lai-Cheong *et al.*, 2008).

6.2 Kindlin regulation of growth factor receptor trafficking

6.2.1 Lysosomal sorting of EGFR

The data in chapter 4 focused primarily on the regulation of EGFR by kindlin-1 as it was shown that EGFR was significantly downregulated at the protein level in KS keratinocytes. It appeared that EGFR was being increasingly degraded in KS keratinocytes via the lysosomal pathway, as inhibition with leupeptin, but not MG132, resulted in increased levels in KS cells. The lysosomal pathway is a well-established mechanism in which proteins are routed and packaged for intracellular degradation. After GFRs are internalised by clathrin-mediated endocytosis, they proceed along the endocytic pathway resulting in downregulation of the receptor (Sorkin and Goh, 2008; Platta and Stenmark, 2011). The endocytic pathway includes the early endosome, late endosome and the terminal lysosome that uses hydrolytic enzymes to digest macromolecules. Those receptors that are not activated are recycled back to the cell surface and are not further targeted for degradation (Platta and Stenmark, 2011). The internalisation of EGFR can be accelerated by acute stimulation with its growth factor ligand EGF, and the adaptor protein Grb2 has also shown to be important for this process (Jiang *et al.*, 2003). Furthermore, EGFR can be targeted for degradation by Cbl proteins which signal the receptor for ubiquitination (Ettenberg *et al.*, 2001). In the case of KS keratinocytes, the addition

of lysosomal inhibitors was shown to rescue the total level of EGFR confirming that it was indeed being degraded in these cells. Given the known synergy between integrins and GFRs, the initial hypothesis was that reduced kindlin-1-mediated integrin activation in KS keratinocytes was responsible for the downregulation of EGFR. However, experiments showed that kindlin-1-W612A was able to rescue EGFR levels and cellular responses to EGF stimulation as well as WT kindlin-1. There was also increased retention of EGFR in kindlin-1-W612A expressing cells, suggesting that EGFR regulation was not integrin-dependent. Moreover, EGFR showed preferential binding to kindlin-1 W612A as opposed to WT kindlin-1, as demonstrated by FLIM analysis, further pointing towards an alternative integrin-independent function of kindlin-1. Taken together, the data suggest that kindlin-1, by binding to EGFR, may retain the receptor to the membrane and prevent its internalisation or that kindlin-1 may act as a signal to prevent EGFR sorting to the degradative pathway. Furthermore, EGF treatment leads to a change in the localisation of kindlin-1 from adhesion sites and an increase in binding to EGFR is also seen after prolonged stimulation. Thus, it appears that kindlin-1 may have a function separate from its role in integrin activation that is involved in the recycling of EGFR and the prevention of its degradation. The mechanism through which this occurs will be an important future area to study and may provide novel insight into cross-talk mechanisms between the two receptor pathways.

6.2.2 Kindlin-1 dependent EGFR recycling

EGFR contains a NPxY motif in the cytoplasmic domain that mediates the interaction with downstream signalling molecules such as Shc (van der Geer *et al.*,

1995). This may also be a potential binding site for kindlin-1 as the latter has already been shown to bind to similar motifs in the cytoplasmic tails of β integrins (Harburger *et al.*, 2009). This NPxY motif is also found in other GFRs such as nerve GFR (NGFR) and IGF-IR as well as in transferrin (TFR) and low-density lipoprotein (LDL) receptors (Hsu *et al.*, 1994; van der Geer *et al.*, 1995). In IGF-IR and transferrin receptors, NPxY has been shown to be an important recognition element for internalisation of the receptor, and mutations introduced in the motif showed that it was also crucial for receptor autophosphorylation and kinase activity (Hsu *et al.*, 1994). Furthermore, the NPxY motif in the LDL receptor was demonstrated to be a binding site for sorting nexin 17 (SNX17). SNX17 has been suggested to play a role in LDL receptor trafficking by sorting the receptor away from the degradative pathway and towards the recycling pathway as it is required for normal endosomal localisation of the receptor (Burden *et al.*, 2004). Interestingly, SNX17 has recently been shown to bind to β1 integrin cytoplasmic tail on the NxxY motif after being recruited to early endosomes to aid its recycling back to the cell surface (Bottcher *et al.*, 2012). Members of the SNX family appear to have differing roles in endosomal trafficking of proteins, for example, they have been identified as mediating EGFR lysosomal degradation, but also show opposing roles in that they can assist recycling of receptors such as transferrin back to the membrane (Kurten *et al.*, 1996). The SNX family, including SNX17, all consist of a phox (PX) domain which facilitates interactions with phosphoinositides, such as those on the endosomal membrane, thereby allowing recruitment to these compartments (Carlton *et al.*, 2005). In addition, SNX17 also posseses a FERM domain similar to that of kindlin and talin in which the PTB like-F3 subdomain has been demonstrated to form interactions with the NxxY motif found on the LDL receptor (Burden *et al.*, 2004). Thus, kindlin-1

appears to possess some features in common with SNX17, and through its FERM domain, it too is able to interact with the same motifs on integrin receptors and, as data from this thesis has shown, potentially on EGFR.

Kindlin-1 also has a PH domain, which raises the possibility that this part of the protein is involved in recruitment to the early endosomal membrane, as occurs with the PX domain in SNX17. The PH domain of kindlin-1 is able to interact with phosphatidylinositol 3,4,5-triphosphate (PIP3), which is found in lipid bilayers of the plasma membrane as well as in the endosomal membrane (Siegel *et al.*, 2003; Yates *et al.*, 2012b). PIP3 functions to promote recruitment and binding of proteins with a PH (or PX) domain. Thus, it is possible that the PH domain drives the recruitment of kindlin-1 to the cell surface and also possibly to the early endosome. Indeed the PH domain has been shown to be important for kindlin-dependent integrin activation as deletion of this domain results in impairment but not total inhibition of integrin activation (Yates *et al.*, 2012b). Studies have also shown that kindlin-2 binds to PIP3 and localises to PIP3-enriched membranes, so it is likely that its family member kindlin-1 also functions in this way (Liu *et al.*, 2011). PIP3 is generated by phosphoinositide-3-kinase (PI3K), which converts phosphatidylinositol 4,5-biphosphate (PIP2) to PIP3. PI3K is activated through EGF stimulation as EGFR can recruit PI3 via adaptor proteins, thus promoting the formation of PIP3 (Bazley and Gullick, 2005). It is feasible, therefore, that kindlin-1 activity is regulated by EGF-mediated PI3K activity and local PIP3 production. Other domains in kindlin-1 also have functional importance. Notably, kindlin-1 has a region homologous to talin within its N-terminus region, known as F0, as well as an insert in its F1 subdomain that has previously not been observed (Goult *et al.*, 2009). The kindlin-1 F0 domain has been shown to adopt a ubiquitin-like fold (Goult *et al.*, 2009) and it has been

suggested that the N-terminal region in kindlins is the site for mediating interaction with ILK and migfilin (Larjava *et al.*, 2008). The insert in the F1 subdomain has been shown to consist of a polylysine motif that allows kindlin to bind to negatively-charged lipid head groups, thereby providing another means by which kindlins can be recruited to the membrane. This domain is also important for targeting to focal adhesions and integrin activation (Bouaouina *et al.*, 2012).

Kindlin-1 may potentially be a downstream signalling molecule of EGF stimulation, as EGFR activation after ligand binding may induce binding of kindlin-1 to its cytoplasmic tail and cause kindlin-1 phosphorylation. Kindlin-1 has previously been shown to be phosphorylated and so it may possibly be a target of intracellular kinase signalling (Herz *et al.*, 2006). EGFR is known to have intrinsic kinase activity, thus presenting a means by which kindlin-1 is phosphorylated. This process may act as a signal for the recycling of EGFR and prevent further targeting for degradation, although it could also serve to recruit kindlin-1 to sites of EGFR localisation at the cell surfaces and away from integrin and focal adhesion sites.

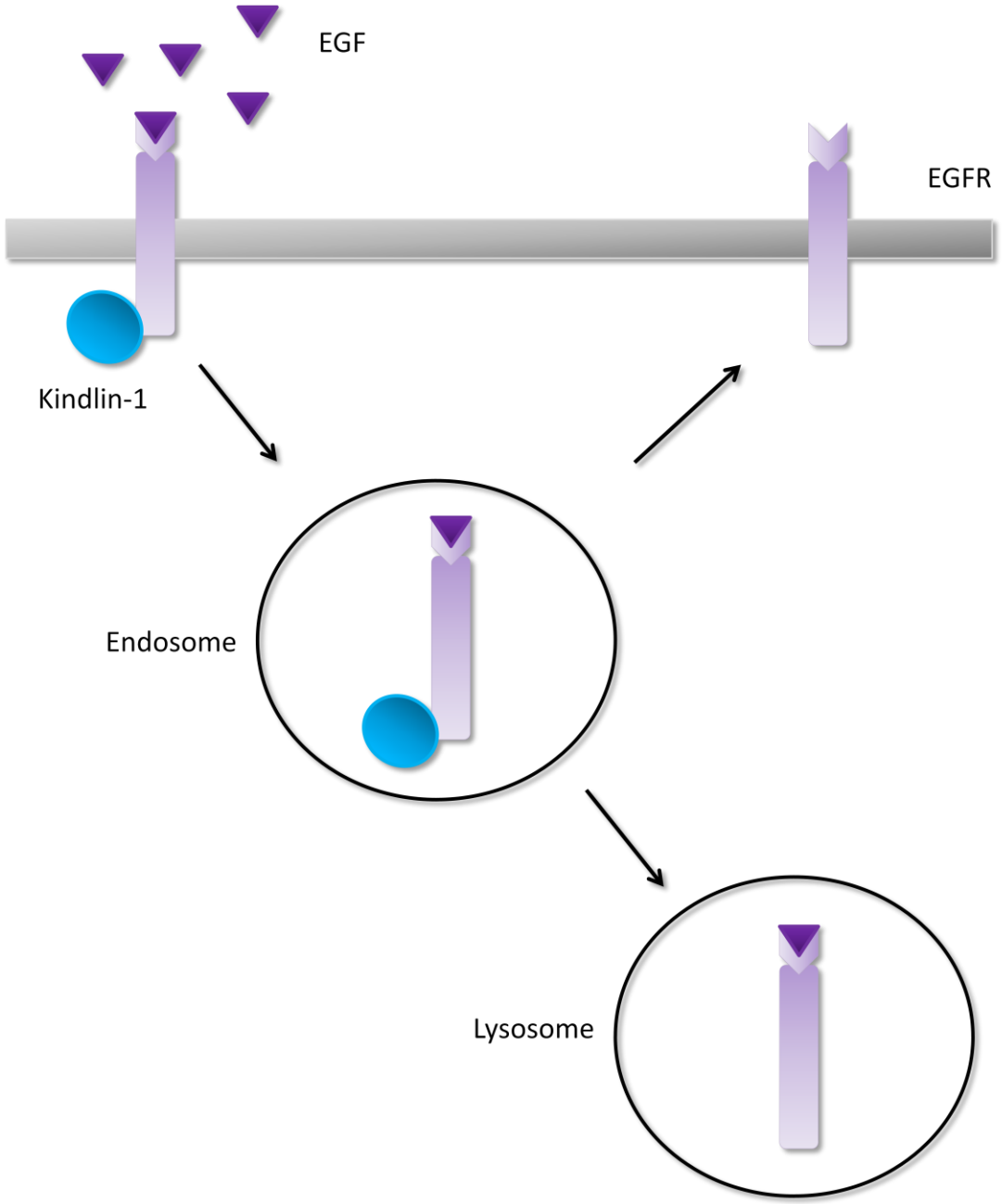


Figure 6.1: Proposed model for recycling of EGFR regulated by kindlin-1

A model showing the recycling pathway of EGFR after stimulation with its ligand EGF. Kindlin-1 may be recruited and bind to EGFR after its activation and is sorted into the early endosome. However the presence of kindlin-1 may prevent further targeting and degradation to the lysosome, and therefore it is recycled back to the cell surface.

6.3 Kindlin-1 and talin interaction

6.3.1 Kindlin-1 mediates talin-dependent activation of integrins

The discovery in this thesis that kindlin-1 is able to interact with talin is a novel finding and raises many new questions regarding potential co-operation between these two integrin activation molecules. Experiments here have demonstrated that kindlin-1 binds specifically to the head (FERM) domain of talin and that this interaction is disrupted when a 9bp KS-patient derived mutation is deleted within the kindlin-1 F3 subdomain. Studies have shown that the talin head domain is key for the activation of integrins as this domain activates when expressed alone (Ma *et al.*, 2008; Montanez *et al.*, 2008; Harburger *et al.*, 2009). However, the same studies have also shown that kindlins are required for the enhancement and full activation of integrins (Ma *et al.*, 2008; Montanez *et al.*, 2008; Harburger *et al.*, 2009). Thus, it appears that talin may be the primary mediator of integrin activation while kindlins primarily assist in this process. The role kindlin-1 plays in binding to talin may possibly be to promote recruitment of talin to the integrin sites or to enable it to localise in a correct position for binding to the NPxY motif on β integrin cytoplasmic tails. Another possibility is that kindlin-1 helps to stabilise talin and prevent its degradation and cleavage via calpain or trypsin by occluding sites of cleavage. The expression levels of talin are upregulated in KS keratinocytes possibly due to compensation effects, and re-expression of both WT kindlin-1 and kindlin-1-W612A was able to significantly reverse the increased protein level of talin. This suggests that kindlin-dependent regulation of talin is independent of kindlin-1 binding to integrins. One potential model might be that kindlin-1-talin binding occurs when kindlin-1 is not bound to β integrin cytoplasmic tail, enhancing the concept that kindlin-1 acts to promote talin-mediated integrin activation. The kindlin-1 9 bp

deletion represents a homozygous germline mutation found in a patient with KS who displayed the full clinicopathological features of this syndrome. This genotype-phenotype correlation suggests that the deletion of the three amino acids encoded by this 9bp deletion is enough to cause significant disruption to the protein, resulting in defective function. The three amino acids in question are isoleucine, glutamic acid and phenylalanine, and are located within the F3 subdomain of the kindlin-1 FERM domain. The biophysical structure of full-length kindlin-1 has yet to be deciphered, although the crystal structure of other FERM domain containing proteins, including talin, have been resolved. The structure of the F3 subdomain of talin interacting with the β integrin cytoplasmic domain has been crystallised, confirming binding to the NPxY motif which previously was only shown biochemically (Anthis *et al.*, 2009). The three amino acids identified as absent in the KS patient and studied in this thesis have not previously been implicated in interaction with integrins. However, the fact that the loss of these residues results in disruption to kindlin-1 function indicates the importance of these specific amino acids. When kindlin-1 is aligned with the protein sequence of talin, similar conserved amino acids are located in a loop after the β strand that is known to be involved in binding to the integrin cytoplasmic tail (Anthis *et al.*, 2009). This location raises the possibility that disruption of the loop, by deletion of these amino acids, could cause changes to the structure or folding of the protein, thus affecting the integrin-binding capability of the protein.

6.3.2 Interactions between FERM domain-containing proteins

Other FERM domain-containing proteins have previously been shown to be able to bind to each other. For example, kindlin-1 and kindlin-2 bind to each other and are

also able to form dimers: the interaction between these two family members has been demonstrated in this thesis and in a previous report, although the functional relevance of this interaction is still unclear (Lai-Cheong *et al.*, 2008). Furthermore, talin has been shown to form interactions with FAK, another FERM domain containing protein. This interaction is mediated through the F3 subdomain of talin and the specific binding site may overlap with that of β integrin (Lawson *et al.*, 2012). Thus, it is possible that the F3 subdomain of talin is also involved in kindlin-1 interaction, as this site is not exclusive for β integrin binding. In addition, talin is also able to form homodimers through its C-terminus rod domain which helps to facilitate actin binding (Gingras *et al.*, 2008). Many FERM-domain containing proteins, such as ezrin, radixin, moesin (ERM) and FAK, are regulated by autoinhibition, whereby the F2 and F3 subdomains bind to the C-terminus regions of these proteins (Smith *et al.*, 2003; Frame *et al.*, 2010). In these cases, autoinhibition maintains the protein in a dormant state and can be activated by phosphorylation, as in the case of ezrin or by the binding of other protein or lipids to the FERM domain, such as with FAK (Smith *et al.*, 2003; Frame *et al.*, 2010). It is possible that a similar mechanism occurs with kindlin-1 and talin, whereby the autoinhibited talin is released for activation by kindlin-1 binding to the talin FERM domain. It is also possible that kindlin-1 offers this function for other FERM domain containing proteins. Kindlin-1 expression is restricted to mostly epithelial cells of the skin and gastro-intestinal tract, while its family member kindlin-2 is ubiquitously expressed, and kindlin-3 is found mostly in haematopoeitic cells. Both of these other kindlins have also shown their important contribution to integrin activation in cooperation with talin, and their deficiency results in severe defects. Both kindlin-2 and -3 also share a similar domain organisation, as well as binding partners, with kindlin-1, and

so it is highly possible that they also carry out similar functions by interacting with talin and facilitating its activity.

6.3.3 EGF mediated regulation of kindlin-1-talin binding

In addition to the role of talin-kindlin binding in integrin activation, there may also be a further link with EGFR and its interaction with talin. Data in this thesis show that EGF stimulation increases the binding of kindlin-1 with EGFR, suggesting a similar mechanism may occur with talin. Nevertheless, it seems unlikely that kindlin-1 forms a complex with talin and EGFR at the same time, especially if the purpose of kindlin-1-talin interaction is to stabilise talin or co-ordinate its correct position for integrin activation. It is more likely that kindlin-1 carries out separate and independent functions with EGFR and talin. This mode of action is supported by the finding that the kindlin-1-W612A non β1 integrin binding mutant preferentially binds to EGFR, showing that when kindlin-1 is not engaged with integrin activation, it instead acts to regulate EGFR activity. How this switch in function between receptor types occurs will be an important area for future studies. Of note, kindlin-1 has previously been demonstrated to be a phosphoprotein (Herz *et al.*, 2006), although the exact residues or the particular kinase responsible for this activity are not known. It is possible that EGFR or downstream signals may regulate the phosphorylation status of kindlin-1 and this in turn may dictate the binding partners through conformational changes or alterations in membrane phospholipid binding.

The ability of growth factors to control integrin activity has been previously demonstrated and ocuurs either through direct activation or the convergence of signals between GFR and integrins (Ivaska and Heino, 2011). Thus, EGF signalling

may be a driving force that regulates integrin and therefore focal adhesion activity, which can influence cell migration and adhesion through the formation of interactions between kindlin-1 and integrin, talin or EGFR. EGF is known to be an important regulating factor in establishing correct cell-cell and cell-substratum contacts in keratinocytes. EGF may control focal adhesions through driving kindlin-1 binding to integrin, talin and EGFR. There is also a possibility that EGF may regulate adherens junctions through kindlin-1 mediated regulation of E-cadherin. Furthermore, there is evidence to suggest that EGF can control hemidesmosome disassembly through phosphorylation of β4 integrin subunit which leads to increased keratinocyte migration (Rabinovitz *et al.*, 2004). Their findings therefore underscore the role of EGF in regulating the activity of several molecules involved in cell-cell and cell-substratum biology.

6.4 Conclusions

The present study has identified several findings that enhance the general understanding of the biological functions of kindlin-1. The results demonstrate that kindlin-1 is involved in the regulation of TSP-1 expression and deposition. Kindlin-1 is also important for the trafficking and recycling of EGFR. In addition, kindlin-1 was shown to be a novel interaction partner of talin, thereby providing new information on how these two proteins may be involved in integrin activation. Collectively, these data have improved the understanding of KS at the molecular and cellular level and the increased knowledge of kindlin-1 and its functional role in novel pathways that subsequently may provide a wider scope for therapeutic

targeting in KS or other aspects of skin biology, perhaps related to cell adhesion, skin ageing and carcinogenesis.

6.5 Future directions

The findings in the project have led to the discovery of novel pathways in which kindlin-1 plays a key role. The ability of kindlin-1 to regulate TSP-1 expression and deposition indicates that it may be involved in varying cellular processes, including angiogenesis. Notably, one of the key clinical features of KS is the presence of small dilated blood vessels, known as telangiectasiae. The aetiology of these aberrant blood vessels is not known but it is plausible that the alterations in TSP-1 in KS may be a contributing factor. However, more information is required regarding the downregulation of TSP-1 and whether it is being increasingly degraded in KS keratinocytes. It is clear that kindlin-1 is essential for this pathway as reintroduction of TSP-1 failed to recover normal deposition of this protein. Integrin activation was also shown to be important for this process, as kindlin-1-W612A non β1 integrin binding mutant failed to rescue TSP-1 levels and normal deposition, while WT kindlin-1 was able to do this. To increase understanding about this pathway further analysis is required, especially of the secretory pathway of TSP-1 in conjunction with integrin activation. Antibodies that recognise active β1 integrin could be used alongside TSP-1 staining to look at the localisation of these proteins and whether there are any differences between control and KS keratinocytes in fixed cells. It would also be interesting to use live cell imaging using tagged proteins of kindlin-1 or β1 integrin and TSP-1 to analyse their dynamics in normal and KS patient cells.

The present study has also shown that kindlin-1 regulates the recycling of EGFR by preventing its degradation, potentially through binding with its cytoplasmic tail. As with TSP-1, kindlin-1 re-expression was sufficient to rescue protein levels and also response to EGF stimulation, although in this case the process was shown to be entirely integrin independent. There are, however, issues that remain unresolved and questions that need to be answered for a clearer conclusion to be made. One of these questions is the mechanism by which kindlin-1 is recruited to the membrane and subsequently how it binds with the EGFR cytoplasmic tail. It is possible that kindlin-1 is recruited to the membrane in an EGF dependent manner and this may be tested by the use of an EGFR tyrosine kinase inhibitor such as AG1478 followed by analysis of kindlin-1 localisation after treatment with EGF. EGF stimulation may actually lead to kindlin-1 phosphorylation, and the examination of the changes in the phosphorylated portion of kindlin-1 by using western blotting or immunoprecipitation after treatment of cells with EGF could provide answers. Moreover, treatment with PI3K inhibitors, such as LY294002, may provide more insight into whether EGF induced changes in Akt signalling or PIP3 production leads to kindlin-1 recruitment to the membrane.

The work in this thesis has provided another clue as to the dynamics of integrin activation by kindlin-1 and talin. There is evidence to show that kindlin-1 is able to form an interaction with talin independently of integrin, although the full significance of this is still unclear. Further investigations will be required to determine the specific subdomains and sequences in talin needed for binding to kindlin-1. This could be facilitated by introducing mutations followed by GST pulldown or immunoprecipitation assays. Alternatively, direct binding assays using WT or mutated purified proteins and surface plasmon resonance analysis could be

performed. However, such an approach may not provide the necessary conformation of each protein that controls binding, and therefore a combination of *in vitro* and in cell analysis should be considered. It would also be interesting to investigate whether kindlin-2 and -3 are able to bind to talin, work that could be achieved using similar approaches taken in this study. Expression of the kindlin-1 9 bp deletion mutant in KS keratinocytes may provide further information about the functional effects on integrin activation, by analysing the level of active integrins with immunofluorescence or FACS. Changes in localisation and dynamic behaviour of talin and the kindlin-1 9 bp deletion mutant could also be determined in these cells with the use of live cell imaging.

Another important question that remains to be determined is whether KS or KS-like conditions could arise as a result of mutations in other genes. EB is known to be caused by mutations in at least 11 different genes that code for structural/signalling components of the hemidesmosome-anchoring filament complex (Shinkuma *et al.*, 2011). Thus far, mutations in *KIND1* represents the only genetic disorder related to skin blistering that is found in focal contacts. It is therefore possible that there could be genetic heterogeneity within the spectrum of KS phenotypes observed and that this could reflect inherent mutations in genes that code for other different components of the focal adhesion complex or indeed some of those proteins identified in this study (e.g. talin). Another aspect to be considered is the role that kindlin-1 may have on other diseases or pathologies. For example, kindlin-1 may make an important contribution to wound healing. Several studies have shown that kindlin-1 deficient cells show a delay in wound healing *in vitro* (Herz *et al.*, 2006; Has *et al.*, 2009). In support of this role, this thesis has shown the importance of kindlin-1 in EGFR regulation and potentially also E-cadherin regulation, both of

which are key proteins involved in single and collective cell migration as well as cell proliferation. Angiogenesis is a process characterised by an increase in angiogenic factors, such as VEGF, and a decrease in anti-angiogenic factors, like TSP-1. The imbalance of TSP-1 levels in cells harbouring kindlin-1 mutations or overexpression may also lead to an increase in angiogenesis, like that seen in primary tumour growth. Thus, it is also possible that deregulation of TSP-1 due to loss of kindlin-1 may have a role in cancer formation. Indeed, patients with KS have been found to have a significantly increased life-time risk of developing malignancies such as cutaneous squamous cell carcinomas (Lai-Cheong *et al.*, 2009b). Kindlin-1 has also been shown to be upregulated in many cancers and this raises the possibility of a further link with EGFR, also known to be upregulated or mutated in many cancers (Weinstein *et al.*, 2003; Bazley and Gullick, 2005). It is possible that dysregulation of kindlin-1 leads to an upregulation of EGFR contributing to a metastatic phenotype. Thus, a better understanding of the functional roles of kindlin-1, as well as other proteins/genes implicated in direct or indirect pathways, may generate fundamental new data on various pathological and physiological processes. The present study has greatly improved the general understanding of kindlin-1 function and has shown that its re-introduction into cells can in some measures rescue the phenotype caused by its loss.

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